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Utilizing the T24-*Ha-ras* transfected MCF10A xenograft model of early human breast cancer progression we have 1) demonstrated the effects of tamoxifen on growth and sequence of progression of human preneoplastic breast disease, 2) validated morphological indices of progression with expression and localization of bcl-2, bax, c-erbB2 and Ki67, 3) established a three dimensional in vitro assay system that recapitulates several important aspects of estrogen-induced growth and preneoplastic progression of MCF10AT1 cells *in vivo*, and demonstrates for the first time the integral role endothelial cells play in ductal-alveolar morphogenesis and proliferation of preneoplastic human breast epithelial cells.

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P K Balakrishnan Reddy 7/14/99

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FINAL PROGRESS REPORT OF WORK ACCOMPLISHED BY P.I. AT KARMANOS CANCER INSTITUTE

BACKGROUND

Design of novel strategies for prevention and cure of breast cancer require an understanding of the molecular and cellular events that characterize the conversion of normal epithelium to precancerous proliferative breast disease (PBD) and then to invasive carcinoma. Elucidation of the mechanisms by which estrogen impacts these processes is critical since a variety of epidemiologic evidence suggests that breast cancer risk is related to the duration of unopposed estrogen exposure during key periods: puberty, postmenarchial, and perimenopausal periods. We proposed to explore this area by using the MCF10AT xenograft model, a novel and unprecedented model for human preneoplastic PBD.

The MCF10AT system is a xenograft model in which the progression of a T24 *Ha-ras* transformed derivative of normal MCF10A, viz., MCF10AneoT (1), can be followed from a histologically precancerous stage to development of invasive carcinoma (2). In contrast to MCF10A cells, MCF10AneoT cells form persistent lesions in immunedeficient mice (2). MCF10AneoT and lines derived by alternating *in vivo* transplantation and *in vitro* culture (MCF10ATn) are collectively known as the MCF10AT system (3). MCF10AT cells grow in nude/beige mice, where over a period of several months, a certain percentage of lesions undergo a sequence of progressive histological changes mimicking those seen in breasts of women at high risk of breast cancer (i.e., atypical hyperplasia, a histological risk factor for development of new or recurrent breast cancer) that culminate in a significant proportion of grafts with frankly invasive carcinoma. The lesions formed by lines of the MCF10AT system are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild hyperplasia (grade 1), moderate hyperplasia (grade 2), atypical hyperplasia (grade 3), carcinoma *in situ* (CIS; grade 4), moderately differentiated carcinoma, and undifferentiated carcinoma (invasive carcinoma; grade 5), as well as histologically normal ducts (grade 0). Thus, the MCF10AT system provides a transplantable, xenograft model of human PBD with proven neoplastic potential (3).

The MCF10AT model is particularly relevant to systematically studying the role of estrogens (and antiestrogens) in the progression of early breast cancer since MCF10AT cells are estrogen receptor positive (ER+) (4,5), a situation that is similar to that observed in women with early breast cancer, and respond to estrogen stimulation both *in vitro* (4,5) and *in vivo* (6). *In vivo* assays have shown that estradiol (E_2) supplementation exerts a direct growth promoting effect on MCF10AT lesions when compared to placebo controls: it not only increases the size, frequency and degree of dysplasia of lesions derived from MCF10AT cells but also accelerates the process of transformation to frank malignancy (6). It must be noted, however, that MCF10AT lesions are characterized as "premalignant" since they never invade into the surrounding host tissues; thus, they provide an ideal system for studying early breast cancer.

Work accomplished in final year

BODY OF REPORT

In vivo assays examining the effect of estrogen on morphological sequence of progression of early breast cancer using MCF10AT1 cells have indicated that the presence of estrogen is necessary to drive the transformation of MCF10AT1 lesions from simple/mild hyperplasia (grade 0/1) observed in placebo-administered control animals to lesions displaying histological characteristics of atypia, CIS and invasive cancer (grades 3-5). These results suggest that MCF10AT1 cells have not undergone all the changes necessary to grow as tumors in nude/beige mice and that exposure to estrogen exerts a promoting effect on lesion growth and neoplastic progression of ER+ human breast epithelial cells (6). Much of this growth promoting effect may be attributed to the effects of estrogen on angiogenesis, since lesions from estrogen-exposed animals differed from placebo controls by dramatic increase in angiogenesis. Immunohistochemical staining of these lesions have indicated good molecular correlation between expression of specific breast cancer genes and degree of dysplasia These markers were selected since their expression levels have been correlated to ER status of human breast cancer cell lines. Bcl-2 protein levels are down regulated in MCF-7 cells that are starved of estrogen, resulting in increased bax/bcl-2 ratio and induction of apoptosis. However, restimulation of estrogen-dependent MCF-7 cells with estrogen results in elevated expression of bcl-2 with a concomitant decrease in bax expression (7,8). Cyclin D1 has been recently shown to form functional complexes with ER in the absence of estrogen (9). This interaction results in transcriptional activation of ER-regulated genes. Cyclin D1/ER interaction is inhibited by antiestrogens, and this interaction is independent of cdk4/cdk6 (9). Expression of high levels of c-erbB-2 has been correlated with poor prognosis (10). Expression of pS2 was examined as it is an estrogen-regulated gene, the expression of which requires a functional ER (11).Bcl-2 staining was observed primarily in simple glandular regions and focally in nonatypical hyperplasia, whereas expression of bax was very weak in all areas of lesions. c-erbB-2 protein expression was absent in simple glands but its expression was increased in higher grades of dysplasia, viz., hyperplasia with atypia, CIS and invasive carcinoma. Cyclin D1 protein expression is negative in all grades except in regions of lesions showing invasive carcinoma where intense nuclear staining of cyclin D1 was observed. pS2 staining was primarily observed in simple and hyperplastic ducts. Statistical analysis of these data by chi squared test indicated significant correlation ($p<0.05$) of expression patterns for bcl-2, erbB-2 and cyclin D1 with degree of dysplasia. Intense nuclear staining of proliferation marker, Ki67, was observed in simple glandular, hyperplasia without atypia, hyperplasia with atypia, CIS and invasive carcinoma. These results indicate that estrogen acts as a mitogen on all grades or stages of preneoplastic progression.

***In vivo* effects of tamoxifen on growth and progression of estrogen-induced MCF10AT1 lesions:** Since we have demonstrated effects of estrogen on growth and progression of preneoplastic ducts arising from MCF10AT1 cells (6), it is important to evaluate the effects of tamoxifen, an antiestrogen that is not only used in treatment of breast cancer but also as a chemopreventive agent. To determine the effects of tamoxifen on growth and progression of

MCF10AT1 cells from the orthotopic site, a total of 10^7 MCF10AT1 cells suspended in Matrigel (Collaborative Research) were injected subcutaneously into the mammary fat pad (orthotopic site) of **ovariectomized female nude mice** that received subcutaneous implants of estradiol (1.7 mg/90 day release) and tamoxifen (5 mg/90 day release, 10 mice) or placebo pellets (5 mice), or **intact sexually mature cycling female nude mice** that received implants of tamoxifen (5 mg/pellet; 17 mice) or placebo pellets (5 mice, two injection sites). Animals were observed twice a week and palpated once a week for lesion formation at the injection site beginning 5 weeks after injection of cells. Mice were sacrificed at 10 weeks after injection by cervical dislocation. National Institutes of Health guidelines for proper and humane use of animals were observed. Tissues from the injection sites were removed, lesions weighed and portions of each fixed in neutral buffered formalin and embedded in paraffin for histological examination. Histological grading of lesions were done as described previously (2,3,6); grade 0, simple epithelium; 1, mild hyperplasia; 2, moderate hyperplasia; 3, atypical hyperplasia; 4, CIS; 5, invasive carcinoma. Each lesion is graded according to the most advanced (deviant from normal) morphological pattern observed within it. All lesions were examined for histological grading and expression of breast cancer susceptibility markers as described before.

Since tamoxifen is being used in clinical trials as a chemoprevention agent, it is important to evaluate its effects on normal mammary gland morphology and function. In order to do this, we have removed mammary glands that did not receive injection of cells from intact controls and tamoxifen-supplemented animals, fixed similarly and analyzed for morphology by (H & E staining) and function (immunohistochemical staining for cytokeratins, bax, bcl-2, and Ki67 for proliferative index).

Results: 80% of lesions derived from control MCF10AT1-injected intact mature cycling female mice showed atypia/CIS whereas 20% displayed invasive cancer. This contrasts with that observed in control ovariectomized animals where 60% of lesions displayed grades 0/1 and only 20% showed atypia (**Fig. 1**). These data indicate that growth and progression of MCF10AT cells are responsive to circulating levels of endogenous estrogen produced in intact cycling female mice. Interestingly, 76% (13/17) of lesions from tamoxifen-supplemented group displayed low or complex hyperplasia, 6% (1/17) of lesions had minimal or focal atypia, 18% (3/17) had invasive carcinoma, and none displayed (0 /17) CIS (**Fig. 1**). These results suggest that the presence of estrogen is essential for preneoplastic progression of MCF10AT1 cells, and that the antiestrogen, tamoxifen though not completely effective in inhibiting proliferation to low and complex grade hyperplasia, causes complete inhibition of neoplastic progression to atypia and CIS (**Fig. 1**). Since, 18% of xenografts displayed invasive cancer (grade 5), these data suggest that maximal effect of tamoxifen is targeted on epithelial cells showing atypical hyperplasia and CIS, i.e., grades that show highest levels of ER, whereas cells displaying invasive cancer (grade 5) express significantly lower or negligible levels of ER probably explaining the lack of efficacy of tamoxifen in inhibiting proliferation of cells exhibiting grade 5 of dysplasia. Alternatively, a subpopulation of MCF10AT1 cells may directly progress from simple/mild hyperplasia (grade 0/1) to invasive cancer (grade 5) without going through atypia and CIS ((Shekhar & Visscher, ms in preparation).

Analysis of effect of tamoxifen on lesion growth and progression of E_2-supplemented

animals showed presence of large lesions that displayed all grades of progression as those observed in lesions derived from the E_2-group. Although the lesions derived from E_2 plus tamoxifen group showed significant apoptosis and comedo necrosis, that probably resulted from tamoxifen, the effects exerted by estrogen pellets (1.7 mg/pellet) on growth and progression of MCF10AT1 cells were dominant, and only very weakly antagonized by dose of tamoxifen used in these experiments.

Histological evaluation of sections cut from mammary glands that did not receive injection of MCF10AT1 cells showed normal ducts with single layer of epithelium and no signs of necrosis. These results indicate the absence of obvious adverse effects of tamoxifen on an otherwise quiescent normal mammary gland.

Effect of tamoxifen on expression of specific breast cancer genes: Lesions harvested from tamoxifen-supplemented and placebo control intact cycling animals were paraffin embedded, sectioned and analyzed by immunohistochemistry for expression of bcl-2, bax, ebb-2 and I-67. **Results**: Expression patterns and levels of these markers correlated well with histological indices recorded from H&E stained sections. Lesions derived from control intact cycling animals showed intense staining for bcl-2 in simple glandular and regions of papillary hyperplasia, and very weak expression of bax. Expression of proliferation marker Ki67 was significantly unregulated as positive nuclear staining for Ki67 was observed in all regions of control lesions, simple, papillary, atypical, CIS and invasive cancer. Ebb-2 expression, though not as intense as that observed in lesions of E_2-supplemented animals, was localized in areas of lesions showing atypical hyperplasia and CIS (Table 1). In contrast, lesions from tamoxifen-supplemented intact cycling animals were negative for ebb-2 expression, weakly positive for bcl-2, and demonstrated strong immunoreactivity to bax antibody that was localized at the tips of papillae and regions displaying invasive cancer. Expression of Ki67 was down regulated which is consistent with the overall low proliferative capacity of tamoxifen exposed lesions (Fig. 1). Although tamoxifen was less effective in blocking progression to invasive cancer, it must be emphasized that areas displaying invasive cancer were very small and focal, and stained negative for Ki67 (Table 1). These data suggest that a subset of MCF10AT1 cells may have bypassed the sequential route of progression and progressed directly from simple glands to invasive cancer; however, once progression to invasive cancer had occurred its ability to proliferate was severely impeded by the presence of tamoxifen (corroborated by negative staining for Ki67 and positive staining for bax).

Comparison of normal mammary glands (that did not receive injection of MCF10AT cells) excised from control and tamoxifen-exposed animals showed no difference in expression patterns of bcl-2, bax and Ki67. Bcl-2 and bax expression were very weak while Ki67 was negative.

Establishment of a three dimensional *in vitro* system that recapitulates *in vivo* aspects of estrogen-induced growth and proliferation Using the MCF10AT1 xenograft model for human proliferative breast disease we have previously demonstrated that E_2 exerts a growth promoting effect on benign or premalignant ductal epithelium by enhancing a) the frequency of lesion formation, b) the size of lesions, c) the speed of transformation from grades 0/1 to grades

3 and higher, and d) the degree of dysplasia (6). Much of this growth promoting effect appears to arise from effects of E_2 on angiogenesis since lesions from unsupplemented animals are either simple or hyperplastic without atypia and lack angiogenesis (6). The dramatic increase in growth and advanced histological grades of progression, concomitant with its remarkable effect on angiogenesis suggests that one of the mechanisms by which estrogen acts as a breast cancer promoter could be through its effect on expression of angiogenesis-regulating factors. Although there is experimental evidence supporting the involvement of angiogenesis in pathogenesis of breast cancer, the exact nature and effects of interaction between human breast epithelial (HBEC) and endothelial cells have not been described thus far. This approach requires an assay system that permits growth and differentiation of both epithelial and endothelial cells.

Results: MCF10A and MCF10AT1-III8 cell lines used in this study are normal or produce preneoplastic lesions, respectively. MCF10AT1-III cells were derived from MCF10AT1 lesions that were harvested from E_2-exposed animals. When MCF10A or MCF10AT1-III8 cells are seeded on reconstituted basement membrane (Matrigel) both lines organize into a 3-D tubular network of cells; however, tubes produced by MCF10AT-III8 cells appear multicellular in contrast to unicellular structures formed by MCF10A cells (**Fig. 2**). When MCF10A or MCF10AT-III8 cells are co-cultured with human umbilical vein endothelial cells (HUVEC) cells on Matrigel, rather than interacting with extracellular matrix, the endothelial cells exhibit preferential adherence to epithelial cells. Although both MCF10A and MCF10AT-III8 cells provide preferential substrate for endothelial cell attachment, only MCF10AT-III8 cells facilitate sustained proliferation of endothelial cells for prolonged periods that are visualized as "endothelial cell enriched spots" (**Fig. 3**) which express factor VIII related antigen and cd31 (**Fig. 4**). At regions of endothelial enriched spots, preneoplastic HBECs undergo ductal-alveolar morphogenesis (**Fig. 3**) that produce mucin, express cytokeratin 8/18 and PCNA (**Fig. 4**). The presence of actively proliferating and functional endothelial cells is essential for supporting ductal-alveolar differentiation of preneoplastic HBECs since without endothelial cells, the epithelial cells formed tubular structures with no alveolar morphogenesis. However, this ability to establish an active angiogenic process and undergo ductal-alveolar morphogenesis is facilitated only by preneoplastic HBECs since normal MCF10A cells fail to sustain similar productive interactions with endothelial cells (**Fig. 3**). The inability of normal MCF10A cells to sustain stable 3-D vascular networks *in vitro* is consistent with its inability to produce persistent lesions in immunedeficient mice (2). Thus, a causal-effect relationship that is mutually beneficial exists between endothelial and preneoplastic HBECs that is critical for generation of functional vascular networks and local proliferative ductal alveolar outgrowths with invasive potential. Both these processes are augmented by estrogen whereas antiestrogens inhibit these processes (**Fig. 3**). Induction and maintenance of angiogenic phenotype is associated with upregulation in expression of interleukin-8 and matrix metalloproteinase-2, and estrogen-induced increase in vascular endothelial growth factor (VEGF) and VEGF-receptor 2 (**Fig. 5**). The three dimensional culture system described provides direct proof of the role of endothelial cells or the need for active angiogenesis in the *de novo* generation of ductal-alveolar outgrowths with tremendous proliferative and invasive potential from single preneoplastic HBECs. This model offers a unique opportunity to study endothelial- and epithelial-cell specific factors that are important for ductal-alveolar morphogenesis, angiogenesis and progression to malignant phenotype (Shekhar *et al*,

manuscript submitted). This assay system is unique from those reported by others in that morphogenesis of ductal-alveolar units resembling terminal ductal lobular units occurs *de novo* (from single cells) rather than from organoids of primary cultures or simple organization of single cells into spherical structures with acini.

Significant accomplishments achieved:

1. We have shown that while MCF10A cells are estrogen receptor (ER) negative, MCF10AT cells are ER positive, i.e., the endogenous ER gene is transcriptionally activated. The activated ER protein in MCF10AT cells is functionally active based on its ability to mediate I) E_2-regulated increase of transcription from both exogenous (ERE-TKCAT), and endogenous E_2 regulated genes, progesterone receptor (1) and pS2 (2), ii) confer estrogen-mediated effects on growth and proliferation. Although our studies thus far have not provided any clues on the mechanisms responsible for transcriptional activation of ER gene, our data do show that it is not a result of 1) alterations in methylation status of ER gene as reported in other ER positive (MCF-7) and ER negative (MDAMB 435) cells, and 2) of positional insertion of exogenous *T24-Ha ras* gene in MCF10AT cells.

Activation of expression of functional endogenous estrogen receptor in MCF10AT xenografts, a model for early human breast cancer. P.V.M. Shekhar, M.L-Chen, J. Werdell, G.H. Heppner, F.R. Miller and J.K. Christman (1998). Int. J. Oncol., **13**:907-915.

2. Results from our studies established the presence of a novel mechanism for functional inactivation of wild type p53 in the absence of genetic alterations. Our data showed not only a correlation between accumulation of conformationally altered wild type p53 and neoplastic behavior of MCF10AT cells, but also that conformationally altered wild type P53 (rather than genetic alterations) is defective in P53-mediated functions such as binding to p53 response sequences and transcription activation.

Altered p53 conformation: a novel mechanism of functional inactivation of wild type p53 in a model for early human breast cancer. P.V.M. Shekhar, R. Welte, J.K. Christman, H. Wang and J. Werdell. Int. J. Oncol., , 11: 1087-1094, 1997.

3. We have demonstrated that the observed epidemiologic link between estrogen and increased risk of breast cancer indeed reflects a direct effect of estradiol on growth and sequence of progression of benign or premalignant ducts. Our results suggest that estrogen exerts a growth promoting effect on benign or premalignant ducts by enhancing a) the frequency of lesion formation, b) size of lesions, c) the speed of transformation from grades 0/1 (simple/mild hyperplasia) to grades 3 (atypical hyperplasia) and higher (carcinoma *in situ* and invasive carcinoma), d) the degree of dysplasia, and e) degree of angiogenesis.

Direct effect of estrogen on sequence of progression of human preneoplastic breast disease. P.V.M. Shekhar, P. Nangia-Makker, S.R. Wolman, L. Tait, G.H. Heppner and D.W.

Visscher. Am. J Pathol, 152: 1129-1132, 1998.

4. We have also demonstrated the estrogenic properties of organochlorine pesticides and its effects on growth and ER function in preneoplastic MCF10AT cells.

Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. P.V.M. Shekhar, J. Werdell and V.S. Basrur. J. Natl Cancer Inst., 89: 174-1782, 1997.

5. We have examined the effect of tamoxifen both as chemotherapeutic and chemopreventive agent. Our results showed that tamoxifen is able to a) block the growth promoting effects exerted by physiological circulating levels of estrogen; b) inhibit progression of MCF10AT lesions to atypia and CIS; c) inhibit proliferation of MCF10AT cells that have progressed to invasive cancer; d) exert maximal inhibitory effects on sequential progression to atypia and CIS, an observation that is consistent with expression levels of ER in DCIS cases of human breast cancer.

Morphological and molecular evaluation of tamoxifen effects on progression of human preneoplastic breast disease. PVM Shekhar, P. Nangia-Makker and D.W. Visscher. Manuscript in preparation

6. We have established a three dimensional (3-D) basement membrane assay system that allows exploration of the interactions between human breast epithelial cells and endothelial cells on reconstituted basement membrane, and show that distinct patterns of angiogenesis permit discrimination between normal (or benign) and premalignant mammary epithelial cells. Results from this study show that estrogen exerts a direct and early effect on mammary carcinogenesis by stimulating proliferation of both endothelial cells (an important stromal component) and premalignant epithelial cells. We demonstrate the existence of a direct causal-effect relationship between endothelial and preneoplastic MCF10AT cells which is integral for generation of active angiogenesis and ductal-alveolar morphogenesis, two processes that are regulated by estrogen at the molecular and cellular levels.

Interaction with endothelial cells is a prerequisite for ductal-alveolar morphogenesis and hyperplasia of preneoplastic human breast epithelial cells: Regulation by estrogen. Malathy P.V. Shekhar, Jill Werdell and Larry Tait. Manuscript submitted.

7. Stabilized existence of P53 in a conformationally altered "mutant" immunological phenotype in MCF10AT xenografts appear to result from its interaction with several high molecular weight proteins present in the milieu of MCF10AT cells. Differences in P53 function between parental MCF10A and MCF10AT xenografts appear to correlate with: 1) absence of phosphorylated native p53 in MCF10AT xenografts, 2) presence of high levels of conformationally altered P53 in MCF10AT xenografts, 3) specific interaction of conformationally altered P53 with high molecular weight proteins in MCF10AT xenografts, 4) interaction of native wild type P53 with MDM-2 possibly resulting in functional inactivation in MCF10AT xenografts. Our results also

show that while MDM-2 associates with a subset of wild type P53 (probably the native form), it clearly does not form complexes with conformationally altered P53. Thus, at least two mechanisms may account for loss of P53 function in MCF10AT xenografts: a) predominant existence of P53 in a conformationally altered state, and b) binding of native wild type P53 with MDM-2. Human breast specimens with known grades and stages of progression have been collected to conduct similar analysis of p53 structure, phosphorylation status and interaction with MDM-2. Results from these studies will establish the existence of similar mechanisms of functional inactivation of wild type p53 in human breast cancers as in MCF10AT cells.

ABSTRACTS PRESENTED

1. Activation of the endogenous estrogen receptor (ER) gene in MCF10AneoT cells, a potential factor in neoplastic progression of MCF10AneoT xenografts. P.V.M. Shekhar, M.-L. Chen, J. Werdell, G.H. Heppner, F.R. Miller and J.K. Christman. Proc. AACR, Vol. 36, 1995 (Poster discussion).

2. Role of p53 in neoplastic progression of MCF10AneoT in a xenograft model for human breast cancer. P.V.M. Shekhar, R. Welte, F. Sarkar and J.K. Christman. Proc. AACR, Vol. 36, 1995.

3. Altered p53 conformation in neoplastic progression of MCF10AneoT xenograft model. P.V.M. Shekhar, J.K. Christman, M.L.-Chen and J. Werdell. Proc. AACR, Vol. 37, 1996.

4. Serial xenograft passage is associated with decreased frequency of N-(phosphonoacetyl)-L-aspartate resistant variants in MCF10AneoT cells. S.K. Kurumboor, P.V.M. Shekhar and J.K. Christman. Proc. AACR, Vol. 37, 1996.

5. Environmental Estrogen Stimulation of Growth and ER Function in Preneoplastic and Cancerous Human Breast Cell Lines. P.V.M. Shekhar, Basrur, V.B. and J. Werdell. Proc. AACR, Vol. 38, 1997 (Poster discussion and News Conference).

6. Estrogen and Proliferative Breast disease. P.V.M. Shekhar. Era of Hope: Sponsored by the Department of Defense in Washington D.C., 1997.

7. Direct effect of estrogen on progression of human proliferative breast disease. Shekhar, P.V.M., Nangia-Makker, P., Wolman, S., Tait, L., Heppner, G.H., and Visscher, D.W. Proc. AACR, 39, 1998.

8. Preneoplastic mammary epithelial - endothelial cell interactions: Regulation by estrogen. P.V.M. Shekhar, J. Werdell, S. Wolman, and L. Tait. Proc. AACR, 40: 2733, 1999. (Poster Discussion).

CONCLUSIONS

1. Our data suggest that maximal inhibitory effect of tamoxifen is exerted on sequential progression of lesions to atypia and CIS, a situation which contrasts with that observed with estrogen where maximal stimulatory effect is exerted on progression to atypia and CIS. Our results also show that within the time frame of our study, tamoxifen does not cause adverse reaction on normal mammary gland.
2. We have established a novel, physiologically relevant *in vitro* model system that not only recapitulates several important aspects of estrogen-induced growth and preneoplastic progression of MCF10AT1 cells *in vivo*, but also demonstrates for the first time the integral role endothelial cells play in ductal-alveolar morphogenesis and proliferation of preneoplastic human breast epithelial cells. Defining the factors and cellular interactions that influence proliferation, invasion, cytological and functional differentiation of mammary epithelial and stromal components such as endothelial cells is key to development of effective intervention strategies for breast cancer.

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APPENDIX

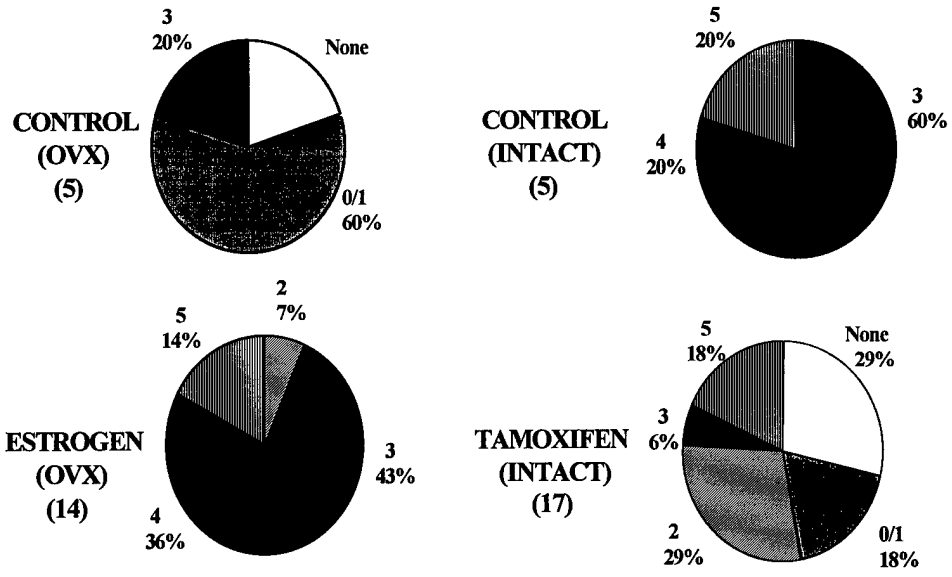


Figure 1 **None - no epithelium; 0/1 - simple, mild hyperplasia; 2 - moderate hyperplasia; 3 - atypical hyperplasia; 4 - CIS; 5 - invasive carcinoma. Intact control group had two injection sites giving rise to ten lesions**

Table 1

IMMUNOHISTOCHEMICAL EXPRESSION OF MOLECULAR MARKERS IN MCF10AT1 LESIONS DERIVED FROM CONTROL AND TAMOXIFEN-EXPOSED CYCLING ANIMALS

GENE PRODUCT	SIMPLE DUCTS	MODERATE HYPERPLASIA	ATYPICAL HYPERPLASIA	CARCINOMA IN-SITU	INVASIVE CARCINOMA
CONTROL					
BCL-2	++	+++	-	-	-
BAX	-/+	-/+	-/+	-/+	-
c-ERBB-2	-	-	+	++	-
Ki67	+	++	++	++	++
TAMOXIFEN					
BCL-2	+	+	-	No CIS	-
BAX	+	++	++	No CIS	+++
c-ERBB-2	-	-	-	No CIS	-
Ki67	-	-/+	-/+	No CIS	-

Figure 2. Phase contrast morphology of cells in 3-D Matrigel culture. (a and b) MCF10A cells at 24 h and 10 days, respectively; (c and d) EIII8 cells at 24 h and 10 days, respectively; (e and f) HUVEC cells at 24 h and 5 days, respectively. Note the difference in contrast on day 10 between MCF10A (b) and EIII8 (d) cultures that is produced by multilayering of epithelium. Bar 100 μm.

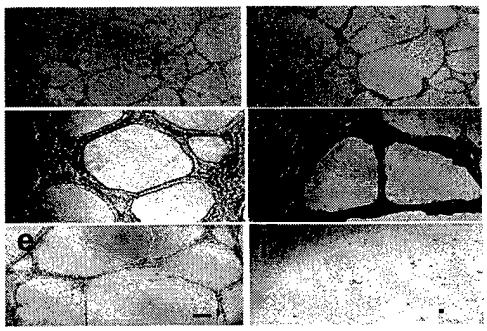


Figure 3. Phase contrast morphology of heterotypic EIII8-HUVEC or MCF10A-HUVEC co-cultures in Matrigel at day 5. MCF10A-HUVEC or EIII8-HUVEC cultures were treated with vehicle (0.01 % ethanol, v/v; panels a and d, respectively), 1 nM E_2, (b and e, respectively), or a combination of 1 nM E_2 and a 100-fold molar excess of ICI 182,780 (c and f, respectively). Note the difference in the sizes of "endothelial cell enriched spots" (⇦) formed on EIII8- and MCF10A-epithelia. Also, note the specific association of "ductal-alveolar outgrowths" (⬅) with "endothelial cell enriched spots" in EIII8-HUVEC cultures (d and e), and the specific inhibition by ICI 182, 780 of ductal-alveolar growth and endothelial cell enriched spots in EIII8-HUVEC (panel f) and endothelial cell growth in MCF10A-HUVEC cultures (panel c). The inset in panel f demonstrates the results of exposure to ICI 182, 780 for 14 days. Bar 100 μm.

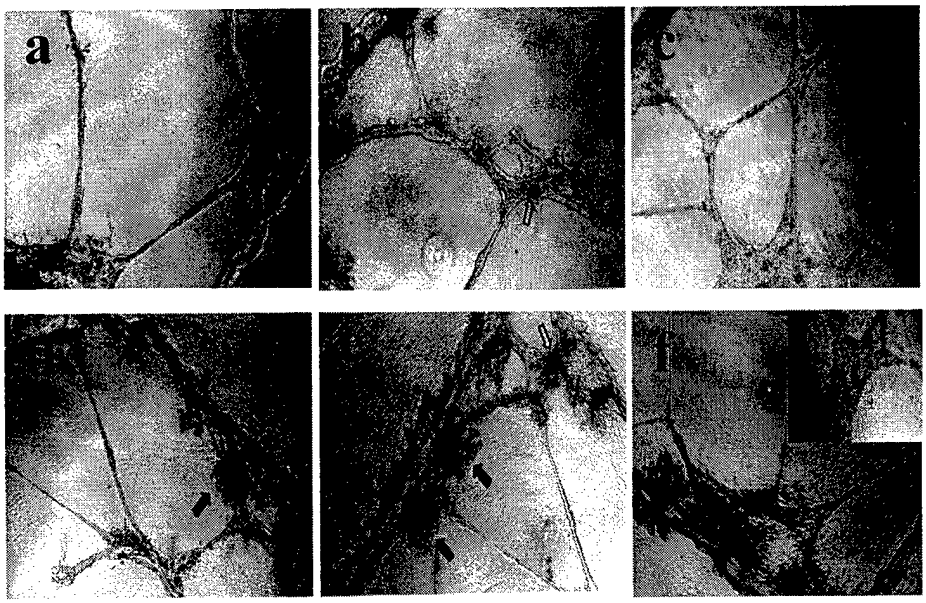


Figure 4. Formalin-fixed paraffin-embedded sections of EIII8-HUVEC 3-D cultures were either stained with H&E (a) or with antibodies to PCNA (b), cytokeratins (c), muc-1 (d), cd31 (e), or Factor VIII (f). Although staining was done on serial sections, the sections look different. This inherent difficulty in obtaining identical serial sections is contributed by the small size and irregular growth of invading branching buds in Matrigel. Note the presence of numerous proliferating cells (b) in the finger-like projections or branching buds that are invading into the surrounding ECM with coincident ECM degradation (a and b). Also, note the widespread immunoreactivity to cytokeratins (c) as compared to the localized distribution of cd31-positive (e; ➤) and Factor VIII-expressing cells (f; ←). MUC-1 staining (d) is predominantly localized in the lumen of epithelium either polarized to apical membrane of luminal epithelial cells or distributed in the cytoplasm (⇦). X10 (a,b,c,e and f) and X 4 (d).

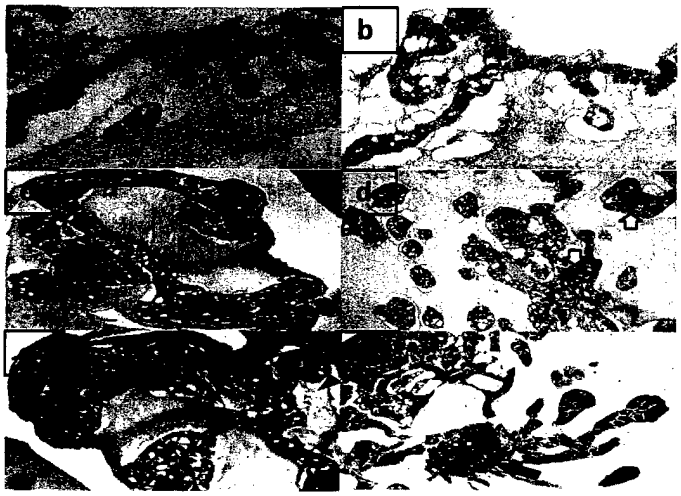
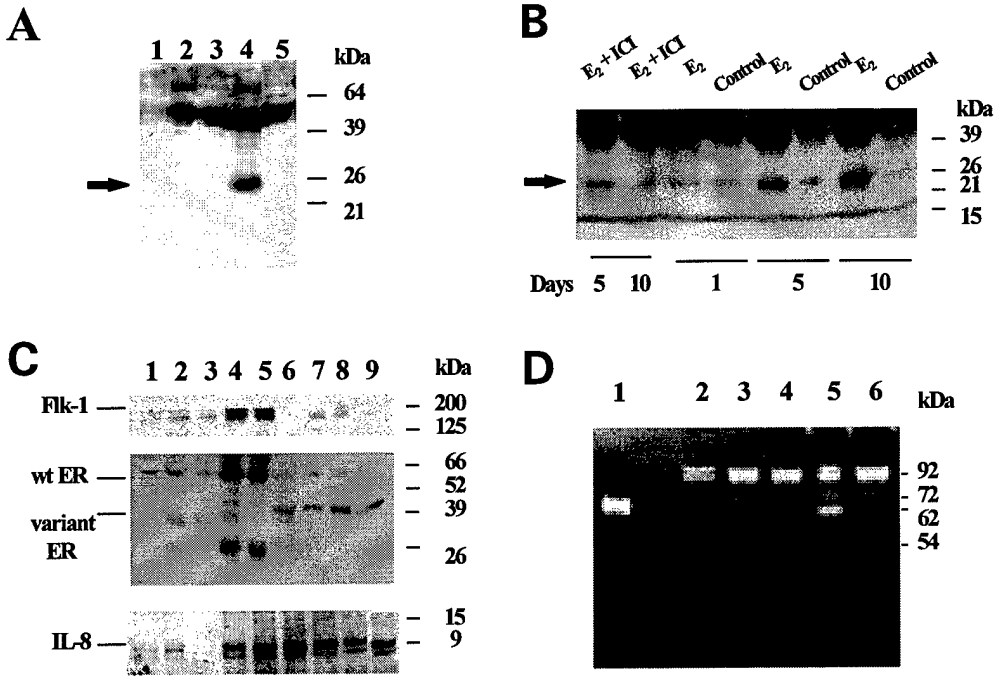


Figure 5. 3-D EIII8-HUVEC co-cultures show upregulated expression of $VEGF_{165}$, Flk-1, ER, IL-8, and MMP-2. 25 μg of protein present in culture media and matrix fractions were analyzed by Western blotting.
(A) $VEGF_{165}$ levels were analyzed in culture media of 5 day-old homotypic (EIII8 or MCF10A), heterotypic (EIII8-HUVEC or MCF10A-HUVEC) cultures, or in HUVEC cells at 48 h of culture. (B) Levels of $VEGF_{165}$ secreted into culture media from EIII8-HUVEC co-cultures were analyzed on days 1, 5 and 10 of culture. Culture media were collected at indicated times from cultures treated with vehicle (0.01% ethanol, v/v), 1 nM E_2 or a combination of 1 nM E_2 and a 100-fold molar excess of ICI 182, 780. Position of $VEGF_{165}$ is indicated by an arrow in panels A and B.
(C) Steady-state levels of Flk-1 and ER, or IL-8 were analyzed in matrix and corresponding culture media, respectively, from HUVEC cultures on day 2 (lane 1), MCF10A-HUVEC (lanes 2 and 3), or EIII8-HUVEC (lanes 4-9) cultures on days 5 (lane 2, and lanes 4-6) and 10 (lane 3, and lanes 7-9). Cultures were treated with vehicle (lanes 1- 4 and 7), 1 nM E_2 (lanes 5 and 8), or with a combination of 1 nM E_2 and a 100-fold molar excess of ICI 182,780 (lanes 6 and 9). Positions of Flk-1, wild type ER, 42 kDa ER-reactive band and IL-8 are indicated.
(D) Zymographic analysis of gelatinases secreted from HUVEC (lane 2), EIII8 (lane 3), MCF10A (lane 4), EIII8-HUVEC (lane 5) and MCF10A-HUVEC (lane 6) cultures. Lane 1 represents activated MMP-2 used as positive control. Conditioned media prepared from 5 day-old EIII8, MCF10A, EIII8-HUVEC or MCF10A-HUVEC cultures, or at 2 days of HUVEC cell cultures were analyzed on gelatin-embedded substrate gels. The gels are representative of three independent experiments.



Interaction with endothelial cells is a prerequisite for ductal-alveolar morphogenesis and hyperplasia of preneoplastic human breast epithelial cells: Regulation by estrogen.

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ABSTRACT

We have previously shown that estrogen-induced preneoplastic progression of *Ha-ras* transformed MCF10A (MCF10AT) xenografts is associated with pronounced angiogenesis. Although there is experimental evidence supporting the involvement of angiogenesis in pathogenesis of breast cancer, the exact nature and effects of interaction between human breast epithelial (HBEC) and endothelial cells have not been described thus far. This approach requires an assay system that permits growth and differentiation of both epithelial and endothelial cells. Here, we report the development of a three-dimensional *in vitro* culture system that supports growth and functional differentiation of preneoplastic HBECs and endothelial cells, and recapitulates estrogen induced *in vivo* effects on angiogenesis and proliferative potential of MCF10AT xenografts. MCF10A and MCF10AT1-EIII8 cell lines used in this study are normal or produce preneoplastic lesions, respectively. When MCF10A or MCF10AT1-III8 cells are seeded on reconstituted basement membrane (Matrigel) both lines organize into a 3-D tubular network of cells; however, tubes produced by MCF10AT-III8 cells appear multicellular in contrast to unicellular structures formed by MCF10A cells. When MCF10A or MCF10AT-III8 cells are co-cultured with human umbilical vein endothelial cells (HUVEC) cells on Matrigel, rather than interacting with extracellular matrix, the endothelial cells exhibit preferential adherance to epithelial cells. Although both MCF10A and MCF10AT-III8 cells provide preferential substrate for endothelial cell attachment, only MCF10AT-III8 cells facilitate sustained proliferation of endothelial cells for prolonged periods that are visualized as

"endothelial cell enriched spots" which express factor VIII related antigen and cd31. At regions of endothelial enriched spots, preneoplastic HBECs undergo ductal-alveolar morphogenesis that produce mucin, express cytokeratin 8/18 and PCNA. The presence of actively proliferating and functional endothelial cells is essential for supporting ductal-alveolar differentiation of preneoplastic HBECs since without endothelial cells, the epithelial cells formed tubular structures with no alveolar morphogenesis. However, this ability to establish an active angiogenic process and undergo ductal-alveolar morphogenesis is facilitated only by preneoplastic HBECs since normal MCF10A cells fail to sustain similar productive interactions with endothelial cells. Thus, a causal-effect relationship that is mutually beneficial exists between endothelial and preneoplastic HBECs that is critical for generation of functional vascular networks and local proliferative ductal alveolar outgrowths with invasive potential. Both these processes are augmented by estrogen whereas antiestrogens inhibit these processes. Induction and maintenance of angiogenic phenotype is associated with upregulation in expression of interleukin-8 and matrix metalloproteinase-2, and estrogen-induced increase in vascular endothelial growth factor (VEGF) and VEGF-receptor 2. The three dimensional culture system described provides direct proof of the role of endothelial cells or the need for active angiogenesis in the *de novo* generation of ductal-alveolar outgrowths with tremendous proliferative and invasive potential from single preneoplastic HBECs. This model offers a unique opportunity to study endothelial- and epithelial-cell specific factors that are important for ductal-alveolar morphogenesis, angiogenesis and progression to malignant phenotype.

INTRODUCTION

Growth and formation of capillary blood vessels or neovascularization is an essential component of solid tumor growth (1, 2). Every increase in the tumor cell population must be preceded by an increase in new capillaries that converge upon the tumor, and this angiogenesis has been directly correlated with tumor growth and metastasis (3). Products derived from both tumor cells and a variety of non-neoplastic mediator systems have been implicated in this vasopoliferative response (2, 4). A number of growth factors, cytokines, and extracellular matrix molecules have been reported to induce or regulate endothelial cell growth or migration *in vitro* (5). These include several well characterized polypeptide growth factors, proteolytic enzymes, interferon, cyclic nucleotides, prostaglandins, heparin, lowered oxygen tension, histamine and other vasoactive amines, and several low molecular weight endothelial mitogens and chemotactic factors (5).

Vascular endothelial growth factor (6) or vascular permeability factor (7) (VEGF/PF) is an endothelial-specific mitogen that mediates developmental, physiological and pathological neovascularization (8). VEGF has been reported to act as a survival factor, preventing the apoptotic death of microvascular endothelial cells (9, 10). The human VEGF gene encodes a dimeric glycoprotein comprising four possible monomers as a result of differential splicing of eight exons that make up the gene product. The four VEGF subtypes are 121-, 165-, 189-, and 206- amino acids in length (11). The smaller forms are secreted whereas $VEGF_{189}$ and $VEGF_{206}$ are retained close to the membrane of producing cells bound to heparan proteoglycans. Receptors

for VEGF, VEGFR-1 (known as Flt-1) and VEGFR-2 (Flk-1/KDR) bind VEGF whereas VEGFR-3 (Flt-4) appears to be specific for VEGF-C (11). Expression of Flk-1/KDR is confined to endothelial cells, accounting for the selective nature of VEGF-induced mitogenesis (11) . VEGF is expressed at high levels in a wide range of tumors and tumor cell lines (12) and is believed to be a key mediator of tumor angiogenesis (13-15) and the high blood vessel permeability characteristic of tumors (16, 17). Expression of VEGF in the uterus has been shown to be rapidly and strongly stimulated by estrogen (18), suggesting that VEGF mediates the normal, estrogen-induced increase in vascular permeability and blood vessel growth in the uterus. Similarly, expression of VEGF is rapidly induced by 17 β-estradiol (E_2) in dimethylbenz(a)anthracene (DMBA)-induced estrogen-dependent mammary tumors (19).

Using the MCF10AT1 xenograft model for human proliferative breast disease we have previously demonstrated that E_2 exerts a growth promoting effect on benign or premalignant ductal epithelium by enhancing a) the frequency of lesion formation, b) the size of lesions, c) the speed of transformation from grades 0/1 to grades 3 and higher, and d) the degree of dysplasia (20). Much of this growth promoting effect appears to arise from effects of E_2 on angiogenesis since lesions from unsupplemented animals are either simple or hyperplastic without atypia and lack angiogenesis (20). The dramatic increase in growth and advanced histological grades of progression, concomitant with its remarkable effect on angiogenesis suggests that one of the mechanisms by which estrogen acts as a breast cancer promoter could be through its effect on expression of angiogenesis-regulating factors.

The extracellular matrix (ECM) acts locally to modulate the responsiveness of endothelial and mammary epithelial cells to external factors. Besides providing a scaffolding during capillary

morphogenesis, the ECM, by virtue of its ability to mediate both biochemical and biomechanical signaling events, has been shown to exert complex local controls on the functions of endothelial cells (21), and growth, differentiation and apoptosis of normal murine and human breast epithelial cells (22, 23). Collagenolytic degradation of endothelial and parenchymal basement membranes is an essential step in the process of tumor invasion and angiogenesis (24). Proteolysis and interruption of the basement membrane and extracellular matrix require the activation of specialized matrix metalloproteinases, the type IV collagenases or gelatinases (MMPs), which degrade basement membrane collagens type IV and V (25). Two species of MMPs, the 72 kDa species (MMP-2, gelatinase A) and the 92 kDa species (MMP-9, gelatinase B), have been cloned and sequenced (25-27). Both MMP-2 and MMP-9 are secreted as latent proenzymes, and require removal of an 80- and 87-amino acid amino terminal domain, respectively, for activation (28, 29).

Using a three dimensional (3-D) basement membrane assay system we demonstrate the existence of a direct causal-effect relationship between endothelial and preneoplastic MCF10AT HBECs which is integral for generation of active angiogenesis and ductal-alveolar morphogenesis, two processes that are regulated by estrogen at the molecular and cellular levels. Furthermore, we show that the abilities to undergo ductal-alveolar morphogenesis and establish an active angiogenic process are dependent on the cellular genotype of the breast epithelial cells, since untransformed parental MCF10A breast epithelial cells lack both these abilities. Finally, our data suggest that active angiogenesis is integral for growth and proliferative potential of "ductal-alveolar structures" which in turn may determine the malignant phenotype.

MATERIALS AND METHODS

Cell lines

The MCF10AT system is a xenograft model of early human breast cancer progression (30). MCF10AneoT cells are T24 *Ha-ras* transformed cells derived from MCF10A human breast epithelial cells (31). MCF10A cells do not form persistent lesions in immunedeficient mice, whereas MCF10AneoT cells do (30). MCF10AneoT and lines derived by alternating *in vivo* transplantation and *in vitro* culture (MCF10ATn) are collectively known as the MCF10AT system (32). The lesions formed by lines of the MCF10AT system are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild to moderate hyperplasia, atypical hyperplasia, carcinoma *in situ* (CIS), moderately differentiated carcinoma, and undifferentiated carcinoma, as well as histologically normal ducts (30). Thus, the MCF10AT system provides a transplantable, xenograft model of human proliferative breast disease with proven neoplastic potential. These studies utilized parental MCF10A cells and the following lines of the MCF10AT xenograft model: MCF10AT1 and MCF10AT1-EIII8. MCF10AT1 represents the first transplant generation of the MCF10AT xenograft model (30). MCF10AT1-EIII8 (referred to as EIII8) cells are breast preneoplastic epithelial cells that were derived from lesions of MCF10AT1 cells arising in estrogen-supplemented animals (20) and respond to estrogen with increased growth *in vitro* and *in vivo*. MCF10A and MCF10AT-derived cells were maintained in phenol red-free DMEM-F12 medium supplemented with 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.02 μg/ml epidermal growth factor (EGF), 100 i.u./ml penicillin, 100 μg/ml streptomycin and 2.5% horse serum. Charcoal stripped serum was not used since it reduces the proliferative capacity and/or viability of MCF10A cells,

possibly due to removal of essential growth factors. The only sera used routinely were those which were unable to support growth of the estrogen-dependent cell line, MCF-7, indicating absence of biologically significant levels of E_2 or other estrogenic compounds.

Human umbilical vein endothelial cells (HUVEC, purchased from American Type Culture Collection) at passage 13 were maintained in Endothelial Serum Free Basal growth Medium (SFM; Gibco BRL Life Technologies, Grand Island, New York) supplemented with EGF (10 ng/ml), basic fibroblast growth factor (bFGF; 20 ng/ml) and fibronectin (10 μg/ml). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. HUVEC cells were used within 10 passages.

Homotypic and heterotypic three-dimensional basement membrane culture of MCF10A, MCF10AT1-EIII8 and HUVEC cells

For homotypic 3-D cultures, $10X10^4$ cells were seeded as a single cell suspension in 8-well chamber slides coated with extracellular matrix of reconstituted basement membrane, Matrigel (Collaborative Biomedical Products, Bedford, Massachusetts), in DMEM-F12-supplemented media for MCF10A cells and its derivatives, or in SFM-supplemented media for HUVEC cells. For heterotypic 3-D co-cultures, $50X10^3$ MCF10A, MCF10AT1, or EIII8 cells were mixed with an equal number of HUVEC cells and seeded onto chamber slides coated with Matrigel as described for homotypic cultures. Typically, heterotypic co-cultures were performed in SFM supplemented with EGF and bFGF since it allows optimal viability, growth and 3-D organization of both HUVEC and MCF10A cells. Slides were incubated at 37°C. Heterotypic co-cultures and homotypic epithelial cell cultures (MCF10A, MCF10AT1, or EIII8) were routinely maintained

up to 3 weeks and morphological development analyzed by phase contrast microscopy.

The interaction between EIII8 and HUVEC cells was determined by prelabeling them with the fluorescent cationic membrane tracers, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 3,3'-dilinoleyloxacarbocyanine perchlorate (DiO; Molecular Probes, Inc., Eugene, Oregon), respectively, prior to co-culturing. These brightly fluorescent dyes diffuse laterally within the plasma membrane resulting in uniform staining of the entire cell, and since transfer of these probes between intact membranes is negligible and cytotoxic effects are minimal, they permit long-term cell tracking in the 3D-cultures.

Preparation of Conditioned media

$50x10^3$ EIII8 or MCF10A cells were seeded alone or mixed with an equivalent number of HUVEC cells on Matrigel in SFM media as described above. Cells were incubated for 6 h to attach and media were replaced with fresh SFM. After appropriate incubation, the culture media from homotypic and heterotypic co-cultures were collected, centrifuged to remove debris and stored at -20°C. Following removal of culture media, matrix containing the 3-D structures was either solubilized for SDS-PAGE and Western blot analysis or fixed in buffered formalin for evaluation of morphology and distribution of functional markers.

Effect of conditioned medium on proliferation of HUVEC cells

$10x10^3$ HUVEC cells were plated in SFM supplemented with EGF, fibronectin and bFGF in 24 well plates. Following 8 h of plating, various volumes of unconcentrated conditioned media prepared from homotypic EIII8 or MCF10A cultures, or heterotypic MCF10A-HUVEC or EIII8-

HUVEC co-cultures were added. For the inhibition experiments, polyclonal antibodies to human VEGF (recognizes the carboxyl terminal epitope found in $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$; Oncogene Science, Cambridge, Massachusetts), mouse monoclonal antibody to human Flk-1/KDR (VEGFR-2; epitope not known; Chemicon International, Inc., Tamecula, California) or polyclonal antibody to human interleukin-8 (IL-8; has <5% cross-reactivity with Groα, Groβ, and Groγ; R&D Systems, Minneapolis, Minnesota) were diluted in SFM and added at 10 μg/ml. Appropriate non-immune IgG was used at 10 μg/ml. Cultures were incubated at 37°C in 5% CO_2-95% O_2 for 5 days with medium change and supplementation of conditioned media or appropriate antibodies every other day. At 5 days of culture, cells were released by trypsinization and viable cells, as demonstrated by trypan blue exclusion, and counted in a hemocytometer. All cell counts were done from triplicate wells and results were expressed as the mean ± standard error (S.E.) from three independent experiments.

Western blot analysis

Analysis of expression of VEGF, IL-8, Estrogen receptor (ER) and Flk-1/KDR proteins was carried out by Western analysis with the specific antibodies. Aliquots of unconcentrated conditioned media or lysates of 3-D cultures containing 20 or 40 μg total protein, respectively, were collected at indicated times of culture, separated by SDS-PAGE on 12.5% (VEGF), 17% (IL-8) or 7% (ER and Flk-1/KDR) polyacrylamide gels (33), and subjected to Western blot analysis. The following antibodies to human proteins were used: rabbit polyclonal anti-VEGF antibody (recognizes the carboxyl terminus present in VEGF165, 189 and 206; Oncogene Science, Cambridge, Massachusetts), mouse monoclonal anti-estrogen receptor (ER) antibody

(Clone 1D5; reacts with the amino terminal domain or A/B region of the receptor; Dako Corp., Carpenteria, California), mouse monoclonal anti-Flk-1/KDR antibody (Chemicon International, Inc., Tamecula, California) and goat polyclonal anti-IL-8 antibody (R&D Systems, Minneapolis, Minnesota). Immunoreactive bands were visualized using the enhanced chemiluminescence detection reagents from Amersham Corp. (Arlington Heights, Illinois), and band intensities quantitated with a Model 300A densitometer (Molecular Dynamics, Sunnyvale, California).

Effects of Estrogen on 3-dimensional growth

To assess growth effects of E_2 on homotypic (EIII8) or heterotypic (EIII8 and HUVEC) 3-D cultures, $50x10^3$ EIII8 cells were seeded alone or mixed with an equivalent number of HUVEC cells in the appropriate medium in 8-well chamber slides coated with Matrigel as described above. Slides were incubated overnight to allow attachment of cells to surface, and treated with vehicle (0.01% ethanol, v/v), pure antiestrogen ICI 182,780 alone (100 or 1000 nM; a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals, Cheshire, U.K.), E_2 (0.1, 1 or 10 nM; Sigma Chemical Co., St. Louis, MO), or a combination of 1 nM E_2 and 100- or 1000-fold molar excess of ICI 182,780. Cultures were incubated at 37°C for 5 days, after which cell viability was measured with the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) kit according to the manufacturer's directions (Promega Corp., Madison, Wisconsin). Measurements were made from triplicate sets of wells for each treatment. Background absorbance was corrected by preparing triplicate sets of wells containing only Matrigel ("no cell") and same volumes of culture medium and MTS reagent as in experimental wells. Average absorbances from "no cell" wells were subtracted from sample absorbance values

containing cells to yield corrected absorbance, and the results represent the average ± S.E. of triplicate samples. Each experiment was repeated at least three times. Since the MTS assays performed here differs from routine MTS assays in that they are done on 3-D cultures growing in Matrigel rather than monolayers, results of MTS assays were validated by performing direct cell counts of viable cells in the 3-D cultures. For this, the medium was removed, wells rinsed with PBS, and Matrigel was digested with dispase for 2 h at 37°C. The digested material was transferred to tubes, centrifuged at 4000 x g for 10 min, and the pellet was rinsed with PBS before treatment with trypsin to recover single cells from the 3-D tubular structures. The number of viable cells was determined by trypan blue exclusion in a hemocytometer. Results were expressed as mean ± S.E. from three independent experiments.

Morphological evaluation

For histological evaluation, 3-D co-cultures were fixed in buffered formalin, embedded in paraffin, and 4 μm sections stained with hematoxylin and eosin (H&E). For immunohistochemical evaluation of mammary epithelial and endothelial cell functional markers, sections were incubated with monoclonal antibodies directed against the following human proteins: pan-cytokeratin 5/6/8/18 is a cocktail of monoclonal antibodies designed to recognize epithelial cells and their tumors (Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.); muc-1 glycoprotein is a mammary type apomucin also known as milk fat globule membrane antigen (Novocastra Laboratories Ltd.); cd31 or PECAM-1 (Dako Corp., Carpenteria, California); Factor VIII related antigen (Dako Corp.), and rabbit polyclonal antibody against proliferating cellular nuclear antigen (PCNA; Dako Corp.). In each instance, negative controls

were overlaid with appropriate mouse or rabbit IgG isotype. The slides were rinsed with PBS and overlaid with avidin-biotin conjugated goat anti-mouse or anti-rabbit IgG (Vectastain ABC kit; Vector Laboratories, Burlingame, California) for 60 min. The slides were washed and incubated for 5 min in peroxidase substrate solution (3,3'-diaminobenzidine), and counterstained by Mayer's hematoxylin (Sigma Chemical Co., St. Louis, Missouri).

Gelatin zymography

The activity of gelatinolytic enzymes in conditioned media of MCF10A, EIII8, HUVEC, MCF10A-HUVEC or EIII8-HUVEC 3-D cultures was detected by electrophoresis in 7.5% (w/v) acrylamide gel co-polymerized with gelatin (Difco Laboratories, Detroit, Michigan) at a final concentration of 0.6 mg/ml. Aliquots of conditioned media containing equivalent amounts of protein were mixed with SDS-sample buffer (33) and electrophoresed without boiling under nonreducing conditions. Following electrophoresis, the gel was soaked for 10 min in 2.5% Triton X-100/10 mM Tris-HCl, pH 8.0 at room temperature, rinsed with 10 mM Tris-HCl, pH 8.0, and incubated at 37°C for 16 h in 5 mM $CaCl_2$/50 mM Tris-HCl, pH 8.0. The gel was stained with 0.1% Coomassie Brilliant Blue R250 and destained. Purified precursor form of Matrix metalloproteinase-2 (pro-MMP-2; gift from Dr. Rafael Fridman, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan) was activated with 1 mM 4-aminophenylmercuric acetate (APMA) and used as a positive control for activated MMP-2 form (34).

Statistical Analysis

Data were analyzed with an analysis of variance. Specific differences among treatments were examined using the Student's t-test. Statistical significance was determined using the Student's t-test with $p<0.02$ considered as statistically significant.

RESULTS

Three-Dimensional Basement Membrane Co-Culture of EIII8 cells with HUVEC cells recapitulate the phenotypic characteristics of preneoplastic breast tissue *in vivo*

Effects of estrogen on 3-D growth of homotypic cultures: When MCF10A cells or *ras*-transformed MCF10AT1 or EIII8 cells are seeded on Matrigel, within 24 h all the lines organize into a 3-D tubular network of cells that are arranged in a duct-like pattern around a central space (Fig. 1, panels a-d). However, profound differences between the duct-like structures of EIII8 cells and MCF10A cells become evident after about 4 days in culture. Tubes produced by EIII8 cells appear multicellular in contrast to the unicellular structures formed by MCF10A cells. The lack of tubular thickening is not due to loss of viability of MCF10A cells as these tubular structures are stable and persist indefinitely as those formed by MCF10AT1 or EIII8 cells. Rather, this difference is due to difference in proliferative capacities of EIII8 and MCF10A cells on Matrigel (Fig. 1, compare panels a and b with c and d) although the two cell lines have similar doubling times (~19 h) on tissue culture plastic (data not shown). Since MCF10AT1 cells exhibit growth characteristics intermediate to those of MCF10A and EIII8 cells, most of our work utilized EIII8 cells. A major difference between MCF10A and EIII8 cells was the growth stimulatory effects of E_2 on EIII8 3-D structures. Treatment with 1 or 10 nM E_2 resulted not only

in marked thickening of tubes over those of control cultures but also in the formation of several new "central spaces" and "connecting bridges" (Fig. 2, compare panels b & d with panel a). These effects could be blocked by a 100-fold molar excess of the pure antiestrogen, ICI 182,780, indicating the specificity of E_2-induced growth effects (Fig. 2, panel c).

In contrast to stable tubular networks formed by the breast epithelial cell lines, similar cultures of HUVEC cells in Matrigel resulted in formation of tubes within 24 h that remained stable only for an additional 48-72 h, and disintegrated by day 5 of culture (Fig. 1, panels e & f).

Heterotypic cultures and effects of E_2 on growth: We compared the abilities of normal MCF10A and preneoplastic EIII8 cells to support and maintain endothelial cell growth. When heterotypic co-cultures of MCF10A or EIII8 cells were set up with HUVEC cells, the tubular networks observed with the homotypic cultures (Figs. 1 and 2) were preserved. However, although equal numbers of epithelial and endothelial cells were seeded, the tubular frameworks were comprised mainly of epithelial cells while endothelial cells demonstrated preferential affinity to attach and proliferate at certain sites of the tubular framework. These regions, referred to as "endothelial cell enriched spots", became prominent at ~ 2-3 days of culture (Fig. 3, panels a, b, d and e). This distribution pattern of the two cell types in the 3-D structures was confirmed in co-cultures of EIII8 and HUVEC cells that were prelabeled with DiI and DiO, respectively (Fig. 4, panels a and b). It is interesting to note that while both normal MCF10A and preneoplastic EIII8 cells provided "soil" for endothelial cell attachment, only EIII8 cells sustained active proliferation of endothelial cells for >3 weeks. This is evident from the remarkable difference in sizes and capillary outgrowths of "endothelial enriched spots" formed between EIII8-HUVEC and MCF10A-HUVEC co-cultures (Fig. 3; compare panels a, b and d, e). The "endothelial cell

enriched spots" present on MCF10A-induced tubular networks remained viable for only ~ one week although the epithelial framework persisted (data not shown). These results suggest major differences in the angiotropic response between normal and transformed human breast epithelial cells; i.e., while MCF10A cells switch from a "pro-angiogenic" to an "anti-angiogenic" phenotype, preneoplastic EIII8 cells remain turned-on in the "pro-angiogenic" state. Another interesting feature unique to EIII8-HUVEC 3-D cultures, and not observed in MCF10A-HUVEC co-cultures, is the development within 2-3 days of co-culture of "branching end buds" or "ductular-alveolar outgrowths" in close proximity with "endothelial enriched spots"(Fig. 3, panels d and e; Fig. 4 a - f). Treatment with 1 nM E_2 enhanced both growth of "endothelial cell enriched spots" and "ductal-alveolar outgrowths" over those of control cultures as seen by an increase in size of both "spots" and ductal branches in 5-day old co-cultures (Fig. 3, compare panels d and e). Although no sera and only phenol red-free media were used, the magnitude and specificity of estrogen-mediated stimulatory effects on angiotropic response and ductal-alveolar morphogenesis/growth became more obvious when cultures were treated with a combination of 1 nM E_2 and a 100-fold molar excess of 4(OH) tamoxifen (data not shown) or the pure antiestrogen ICI 182,780. By day 5, these cultures showed dramatic inhibition of both endothelial sprouting and associated ductal morphogenesis (Fig. 3 f) that disintegrated by day 10-14 of culture (Fig. 3f inset). These data suggest that the presence of contaminating estrogen in the culture media probably contributed to endothelial cell growth and ductal-alveolar morphogenesis observed in control cultures (Fig. 3 d). It is interesting to note that although addition of estrogen to MCF10A-HUVEC co-cultures had no significant influence on growth and proliferation of endothelial or epithelial cells (Fig. 3 a and b), addition of ICI 182, 780 abolished formation of "endothelial

enriched spots" while maintaining the MCF10A epithelium (Fig. 3 c).

Quantitative assessment of estrogen effects on 3-D growth of heterotypic (EIII8-HUVEC) cultures

Since exposure to E_2 confers pronounced growth enhancement of EIII8 cells both in homotypic and heterotypic cultures (Figs. 2 and 3), we measured the effects of E_2 on cell proliferation in heterotypic 3-D co-cultures by both MTS and trypan blue dye exclusion assays of dispase-treated cultures. By both assays, E_2 elicited a dose dependent induction of growth at concentrations > 1 nM; a 2-fold increase in growth over control cultures was observed with 10 nM E_2 ($p<0.01$; Fig. 5). This induction of growth by E_2 occurs through the ER-dependent pathway, since the proliferation effects of E_2 were abolished by inclusion of 100-fold molar excess of the pure antiestrogen, ICI 182,780 ($p<0.001$; Fig. 5). Only the results of MTS assay are shown in Fig. 5, since results of both assays were in good agreement.

Only conditioned media from heterotypic co-cultures have endothelial cell growth stimulatory activity

To determine whether the stimulatory effects on endothelial cell proliferation observed in EIII8-HUVEC cell co-cultures are due to soluble factor(s) secreted by EIII8 cells, we examined the effects of conditioned media prepared from homotypic (EIII8 or MCF10A) or heterotypic (EIII8-HUVEC or MCF10A-HUVEC) 3-D cultures on HUVEC cell proliferation. Addition of up to 50 µl of conditioned media from homotypic EIII8 (Fig. 6), MCF10A (data not shown) or heterotypic MCF10A-HUVEC (Fig. 6) cultures were ineffective in stimulating growth of

HUVEC cells over that of control cultures. In contrast, conditioned media from heterotypic EIII8-HUVEC co-cultures elicited a significant dose dependent induction of growth at volumes greater than 5 μl ($p= 0.02$), and addition of 20 μl of conditioned medium elicited a 3-fold increase in HUVEC cell proliferation over that of control cultures ($p<0.001$; Fig. 6). These results indicate that secretion of growth stimulatory activity into the culture media requires intimate interaction between epithelial and endothelial cells. Since the cytokine, IL-8 (35-38), and the endothelial specific mitogen, VEGF, have been demonstrated to function as survival and anti-apoptotic factors for endothelial cells, we tested the effects of antibodies to VEGF, its receptor, Flk-1/KDR, and IL-8 on conditioned media-induced growth of HUVEC cells. Since expression of Flk-1/KDR is confined to endothelial cells, effects of neutralization of Flk-1/KDR with its antibody would indicate the selective nature of VEGF-induced mitogenesis. Addition of 10 μg/ml of antibodies to either VEGF or its receptor, Flk-1/KDR, abolished conditioned media-induced HUVEC cell proliferation ($p<0.001$), whereas addition of similar amounts of polyclonal anti-human IL-8 antibody evoked only 40% inhibition of growth ($p<0.02$; Fig. 6). Inclusion of equivalent amounts of the corresponding normal IgG had no effect on conditioned media-stimulated growth (Fig. 6). This inability of anti-IL-8 antibody to evoke a greater degree of endothelial cell growth inhibition is not due to incomplete neutralization of IL-8 in the conditioned media since addition of higher amounts (up to 25 μg/ml) of IL-8 specfic antibody failed to increase the magnitude of growth inhibition (data not shown). Our results suggest that although IL-8 is an important endothelial cell survival factor in our assays, it is not as potent as VEGF. Support for this assumption is also provided by the equally potent inhibition of growth by anti-Flk-1/KDR antibody as by VEGF antibody.

Heterotypic EIII8-HUVEC 3-D structures express epithelial and endothelial cell function markers

Results of Figs. 3, 4 and 6 clearly demonstrate that in heterotypic EIII8-HUVEC co-cultures, not only do both endothelial and epithelial cell populations remain proliferative but that there is an intimate interaction that is mutually beneficial. This is evident from the co-localization of branching "alveolar outgrowths" with "endothelial cell enriched spots" (Figs. 3 and 4). Histologic evaluation of H&E stained paraffin-embedded sections of 3-D co-cultures revealed the presence of multi-layered epithelium at several regions of the tubular framework with branching end buds (resembling finger-like projections) invading into the surrounding ECM with coincident ECM degradation (Fig. 7 a). In order to confirm the proliferative potential of the 3-D structures and to provide biochemical evidence for breast epithelial and endothelial cell growth and function, we utilized immunochemistry to examine the distribution of epithelial (cytokeratins, muc-1), endothelial (cd31, factor VIII related antigen), and proliferation (PCNA) markers in 10-day old co-cultures. While positive cytoplasmic immunoreactivity to pan-cytokeratins was observed in the majority of cells as expected (Fig. 7 c), muc-1 glycoprotein expression was predominantly localized in the lumen or lumen-forming areas of epithelium either polarized to apical membrane of luminal epithelial cells or distributed in the cytoplasm (Fig. 7 d). These data confirm that the main tubular network is indeed comprised of breast epithelial cells that in 3-D cultures synthesize and secrete mucin, a characteristic feature of epithelial glandular differentiation (39). In contrast to widespread distribution of cytokeratin immunoreactivity, reactivity to the endothelial cell marker, cd31, is restricted to areas of the tubular framework that appears to correspond with "endothelial cell enriched spots" (Fig. 7 e).

Expression of Factor VIII related antigen is localized to "endothelial cell enriched spots" whereas the epithelial branching end buds and tubular framework are negative (Fig. 7f). It is interesting to note that majority of nuclei demonstrate positive nuclear immunoreactivity to anti-PCNA antibody, corroborating the high proliferative capacity of the cells in the 3-D structures (Fig. 7 b).

Expression of VEGF, IL-8, ER and Flk-1 proteins is up-regulated in EIII8-HUVEC cell co-cultures

We have shown that unlike normal MCF10A cells, preneoplastic EIII8 cells provide a "good soil" for optimal survival, proliferation and functioning of endothelial cells. This ability appears to be facilitated at least in part by secretion of endothelial cell growth stimulatory factors, VEGF and IL-8, into the culture media (Fig. 6). Densitometric analysis of the steady-state levels of VEGF protein in the culture media of homotypic (EIII8, MCF10A or HUVEC) and heterotypic (EIII8-HUVEC or MCF10A-HUVEC) 3-D cultures showed the presence of ~20-fold higher levels of $VEGF_{165}$ in culture media of EIII8-HUVEC cultures when compared to corresponding fractions from homotypic EIII8 or MCF10A, or MCF10A-HUVEC cultures (Fig. 8 A). Since the antibody used for Western analysis recognizes the carboxyl terminal epitope that is present in $VEGF_{165,}$ $VEGF_{189}$ and $VEGF_{206}$ but not in $VEGF_{121}$ (11), alterations in relative levels of $VEGF_{121}$ have not been determined. Densitometric analysis of $VEGF_{165}$ in culture media of EIII8-HUVEC 3-D cultures at various times of culture revealed that while control samples maintain a constant level of $VEGF_{165}$ from days 1 to 10 of culture, treatment with 1 nM E_2 induces 4- to 8-fold increase in $VEGF_{165}$ levels over those of control cultures by day 5 and 10 of culture, respectively

(Fig. 8 B). The role of estrogen in regulation of VEGF expression became more apparent when addition of pure antiestrogen, ICI 182,780, at 100-fold molar excess of E_2 abolished the E_2-stimulated increase in $VEGF_{165}$ levels, returning them to control levels (Fig. 8 B). Analysis of expression of the cytokine, IL-8, in culture media of HUVEC (Fig. 8 C, lane 1) or MCF10A-HUVEC 3-D cultures (Fig. 8 C, lanes 2 and 3) showed the presence of only trace amounts of IL-8. In contrast, levels of IL-8 are significantly up-regulated (>10-fold) in culture media of corresponding 5- and 10-day old EIII8-HUVEC co-cultures (Fig. 8 C, lanes 4 and 7). Interestingly, loss of IL-8 expression in 10-day old MCF10A-HUVEC co-cultures (Fig. 8 C, lane 3) is coincident with loss of viability and stability of endothelial enriched sites on MCF10A epithelium. In contrast to the inductory effects of estrogen on VEGF expression, E_2 does not appear to regulate IL-8 expression (Fig. 8C, lanes 5 and 8), and addition of 100-fold molar excess of the pure antiestrogen, ICI 182,780, had no effect on IL-8 levels (Fig. 8 C, lanes 6 and 9).

Analysis of levels of Flk-1/KDR and ER proteins in the corresponding matrix fractions of homotypic (HUVEC) and heterotypic (MCF10A-HUVEC or EIII8-HUVEC) 3-D cultures, revealed the presence of the endothelial cell-specific receptor, Flk-1/KDR, in HUVEC cells as well as in MCF10A-HUVEC and EIII8-HUVEC co-cultures (Fig. 8 C). However, levels of Flk-1/KDR protein are significantly upregulated (~ 12-fold) in 5-day old EIII8-HUVEC co-cultures (Fig. 8 C, lane 4) over those expressed in corresponding MCF10A-HUVEC co-cultures (Fig. 8 C, lane 2) or homotypic HUVEC cultures (Fig. 8 C, lane 1). Although the levels of Flk-1/KDR protein in control and E_2-treated 5 day-old EIII8-HUVEC cultures appear to be equally up-regulated (Fig. 8 C, lanes 4 and 5), addition of ICI 182, 780 at 100-fold molar excess of E_2

caused ~80% reduction in Flk-1/KDR protein levels (Fig. 8 C, compare lanes 5 and 6). While the levels of $VEGF_{165}$ (Fig. 8 B) and IL-8 (Fig. 8 C) proteins remained elevated in 5 and 10-day old EIII8-HUVEC cultures, Flk-1/KDR protein levels in 10-day old EIII8-HUVEC co-cultures declined by ~75% (to levels observed in 10-day old MCF10A-HUVEC co-cultures; Fig. 8 C, lane 3), and were no longer regulated by E_2 or ICI 182,780 (Fig. 8 C, lanes 7-9). It is interesting to note that the pattern of expression of Flk-1/KDR protein parallels the expression pattern of ER in EIII8-HUVEC co-cultures. HUVEC cells are ER-positive (40), whereas MCF10A cells are ER-negative (41), and as expected, a constant level of wild type ER protein (67 kDa) probably originating from HUVEC cells is detected both in homotypic HUVEC (Fig. 8 C, lane 1) and heterotypic MCF10A-HUVEC 3-D cultures (Fig. 8 C, lanes 2 and 3). As observed in the case of Flk-1/KDR protein expression, levels of wild type ER protein are enhanced ~25-fold in heterotypic EIII8-HUVEC co-cultures relative to those in HUVEC or MCF10A-HUVEC cultures (Fig. 8 C, lanes 4 and 5). Treatment with ICI 182, 780 at 100-fold molar excess of E_2, resulted in significant reduction in amounts of wild type ER with concomitant appearance of a 42 kDa protein that is immunoreactive with the anti-ER antibody (Fig. 8 C, compare lanes 5 and 6). It is not yet clear whether the 42 kDa (Fig. 8 C, lanes 6-9) and the 26 kDa bands (Fig. 8 C, lanes 4 and 5) represent variant or truncated forms of ER (42). In 10-day old EIII8-HUVEC co-cultures, the 67 kDa wild type ER band is either undetectable or present in only trace amounts whereas the 42 kDa band represents the major immunoreactive ER band (Fig. 8 C, lanes 7-9). It is not yet clear whether this shift in ER protein expression from 67 kDa to 42 kDa species reflects an alteration in hormonal sensitivity or responsiveness of the EIII8-HUVEC 3-D cultures.

Expression of an active form of MMP-2 is enhanced in EIII8-HUVEC co-cultures

Degradation and remodelling of ECM are essential processes for angiogenesis and involve the matrix metalloproteinase/tissue inhibitor of metalloproteinases (MMP/TIMP) family of proteases (43-45). MMP-mediated matrix remodelling also appears to promote the growth of tumor cells, possibly by facilitating the proliferation and migration of endothelial cells and the neovasculature of tumor tissue (46). Results of histological evaluation of 3-D cultures have shown that in the presence of endothelial cells, EIII8 cells have acquired the ability to invade and degrade the surrounding ECM (Fig. 7, panel a). MMPs including MMP-2 (47, 48) and MMP-9 (49, 50) have been shown to play major roles in degradation of ECM in tumor invasion. In order to identify the gelatinolytic activity of 3-D cultures, we performed gelatin zymography of conditioned media from homotypic (HUVEC, EIII8 or MCF10A) and heterotypic (EIII8-HUVEC or MCF10A-HUVEC) cultures. While the latent or pro-form of MMP-2 (72 kDa) was detected in all samples at low levels, the active form of MMP-2 (62 kDa) was present at significantly elevated levels only in the conditioned media of EIII8-HUVEC co-cultures (Fig. 8 D, lane 5). No difference in the activities of proteins corresponding to 92 (MMP-9) and 96 kDa bands was observed (Fig. 8 D). This lack of regulation of MMP-9 levels suggests either the presence of contaminating MMP-9 that is secreted from Matrigel during culture (51) or a less important role for MMP-9 in interactions between EIII8 and HUVEC cells in our system.

Discussion

Despite wide agreement about the involvement of estrogen in the etiology of breast cancer, there is uncertainty as to its precise role(s) in the biology of breast cancer development. In this report, we

describe an *in vitro* assay system that allows exploration of the interactions between human breast epithelial cells and endothelial cells on reconstituted basement membrane, and show that distinct patterns of angiogenesis permit discrimination between normal (or benign) and premalignant mammary epithelial cells. Results from this study show that estrogen exerts a direct and early effect on mammary carcinogenesis by stimulating proliferation of both endothelial cells (an important stromal component) and premalignant epithelial cells. This assay system is unique from those reported by others in that morphogenesis of ductal-alveolar units resembling terminal ductal lobular units occurs *de novo* (from single cells) rather than from organoids of primary cultures or simple organization from single cells into spherical structures with acini. When normal MCF10A or preneoplastic MCF10AT1-EIII8 cells are co-cultured with HUVEC cells on a layer of Matrigel, all cell types in both co-cultures rapidly organize into interconnected tubes with the endothelial cells preferring to grow on the epithelium. However, although further differentiation of endothelial cells into complex 3-D networks is observed in both MCF10A- and EIII8-HUVEC co-cultures at approximately two days of culture, stable and sustained proliferation of endothelial networks is observed only in EIII8-HUVEC co-cultures. These events are reminiscent of the process of vasculogenesis *in vivo*, where endothelial cells differentiate, proliferate and coalesce to form a developing vascular network or plexus. Subsequently, this network is transformed into mature 3-D vascular networks through angiogenic remodelling processes that involve sprouting, branching and differentiated growth of blood vessels (52). Although the endothelial cells grow on top of epithelial cells, stable and functional 3-D vascular networks (established by Factor VIII expression) develop only at specific sites on the EIII8 epithelium. It is not yet clear whether the occurrence of 3-D vascular networks at

specific sites is induced by a subpopulation of EIII8 cells (with morphological, genetic or functional alterations) that are committed progenitors of cancers. This is possible since like its parental line, MCF10AT1, lesions arising from EIII8 cells in immunedeficient mice exhibit a heterogeneous spectrum of advanced histological grades of progression (atypical hyperplasia, CIS and invasive carcinoma) besides simple ducts, with prominent angiogenesis (Shekhar PVM and Visscher DW, manuscript in preparation). The inability of normal MCF10A cells to sustain stable 3-D vascular networks *in vitro* is consistent with its inability to produce persistent lesions in immunedeficient mice (30).

The onset of malignancy is an active process that requires reciprocal paracrine interactions between endothelial and tumor cells (53). Data from our *in vitro* assay exemplify the need for close and cooperative paracrine interactions at a very early stage between preneoplastic mammary epithelial cells and endothelial cells in the generation of both an angiogenic response and ductal-alveolar morphogenesis. We show that preneoplastic breast epithelial cells facilitate active proliferation and development of stable endothelial cell-derived 3-D vascular networks via soluble angiogenic factors such as VEGF and IL-8, and the 3-D vascular networks or "sustained angiogenesis" in turn assist in generation of local "ductal-alveolar outgrowths". It must be noted that although "sustained angiogenesis" induces formation of alveolar outgrowths, an indicator of mammary glandular differentiation (established by morphology and muc-1 expression), these outgrowths are not normal as they have tremendous potential to proliferate and invade/degrade the surrounding ECM. These observations sharply contrast with those between normal MCF10A and endothelial cells, where no such productive reciprocal interactions occur. These data indicate that the epithelial cell background plays a major role in expression and maintenance of the

angiogenic phenotype.

One of the factors that may contribute to the observed differences in angiogenic response between normal MCF10A and preneoplastic MCF10AT1-EIII8 cells is the presence of activated *Ha-ras* in the latter (31). Tumor angiogenesis is regarded as a highly regulated process that is controlled genetically by alterations in oncogene and tumor suppressor gene expression and physiologically by the tumor microenvironment (54, 55). Previous studies have indicated that the angiogenic switch in *ras*-transformed cells may be physiologically promoted by the tumor microenvironment through induction of the angiogenic mitogen, VEGF. Although our data support the role of activated *ras* in sustaining angiogenesis, it is clearly not sufficient since all MCF10AT cells stably express activated *ras,* yet the development of stable 3-D vascular networks occurs only at specific sites on the EIII8 epithelium.

Another important difference between MCF10A and MCF10AT cells that may contribute to observed differences in angiogenic response is the expression of functional wild type ER in MCF10AT cells (56, 57) whereas MCF10A cells are ER-negative (41, 58). We have previously shown that estrogen enhances preneoplastic progression of MCF10AT1 cells *in vivo* as lesions of MCF10AT1 (20) or EIII8 cells (Shekhar PVM and Dan Visscher, manuscript in preparation) harvested from estrogen-supplemented animals exhibit rapid growth and advanced histological grades of progression, i.e., atypical hyperplasia, CIS and invasive carcinoma with prominent angiogenesis as compared to simple or moderate hyperplasia without atypia or angiogenesis in control unsupplemented animals (20). The importance of estrogen in regulation of the angiogenic response *in vivo* is recapitulated in our *in vitro* assays, since estrogen specifically stimulates growth of both 3-D vascular networks and ductal-alveolar outgrowths, and these

processes are blocked or significantly inhibited by the pure antiestrogen, ICI 182,780.

Endothelial cells have been found to possess ER (40) and estrogen has been reported to increase endothelial cell proliferation (59). In our system, much of the E_2/ER-mediated effects on angiogenesis appear to emanate from its stimulatory effects on expression of angiogenesis regulating factors, VEGF and its receptor, Flk-1/KDR (VEGFR-2). These data are consistent with previous reports that estrogens influence VEGF/PF mRNA expression in the uterus (18), and in the well-vascularized, DMBA-induced hormone-dependent rat mammary tumors (60). Fukeda *et al.* (61) observed that the growth of capillary endothelial cells in the DMBA-induced tumors is estrogen-dependent, and that treatment of DMBA-exposed rats with E_2 after ovariectomy prevents tumor necrosis and maintains high rates of endothelial cell proliferation. Similarly, estrogen-induced angiogenesis in rat pituitary tumors is associated with E_2-mediated increases in the expression of both ligand and its receptor, VEGF and Flk-1/KDR, respectively, suggesting an important role for estrogen in the initial step of angiogenesis regulation (62). In contrast to effects of estrogen on VEGF and Flk-1/KDR expression in our system, steady-state levels of the cytokine, IL-8, an important endothelial cell survival factor, are unaffected by estrogen and remain elevated through out the culture period. A similar lack of regulation by estrogen of several cytokines, including IL-8, has been reported in cells of the human osteoblast lineage (63).

It is interesting to note that in 10-day old cultures, although high levels of IL-8 and $VEGF_{165}$ proteins are maintained, Flk-1/KDR protein levels are significantly reduced but maintained at a steady level that is unaffected by exposure to either estrogen or ICI 182,780. Although the significance of this is not yet clear, this alteration in regulation of Flk-1/KDR protein is

correlated with a dramatic downregulation in levels of the 67 kDa wild type ER and concomitant appearance of a prominent 42 kDa anti-ER immunoreactive protein. It remains to be established whether the 42 kDa protein represents a form of variant ER or is simply a protein that is cross-reactive with amino terminus specific anti-ER antibody. A plethora of studies have reported the detection by RT-PCR of ER mRNA splice variants in normal and cancerous human breast tissues, the biological and clinical significance of which might be significant but remain to be established because of a lack of evidence for their existence at the protein level (64, 65). However, it is intriguing to speculate that such a shift in ER status, coupled with alterations in sensitivity of Flk-1/KDR expression to estrogen and ICI 182,780, may signify a switch from an estrogen-responsive to an estrogen-insensitive phase of angiogenesis and mark the beginning of "new autonomous growth".

Expression of angiogenic activity is a predictable property of many preneoplastic cells and may represent one of the earliest indication that a cell population has become committed to malignancy (66). Results from our novel *in vitro* assay system reinforce the concept that like tumor cells, preneoplastic breast epithelial cells actively produce diffusible angiogenic factors and cytokines that directly activate endothelial cells stimulating them to sprout and initiate development of 3-D vascular networks which in turn induce development of ductal-alveolar outgrowths with capacity to invade and degrade the surrounding ECM. The characteristic expression of activated MMP-2 observed only in preneoplastic epithelial-endothelial cell cultures fortifies the importance of proteolytic enzymes in the release of angiogenic factors sequestered in ECM (67). Matrix proteins such as laminin, fibronectin and collagen often contain bFGF sequestered in the ECM complexed to heparin sulphate proteoglycans (67, 68). The direct

correlation observed between growth and development of vascular networks and alveolar outgrowths with coincident ECM remodeling suggests that increased gelatinolytic activity secreted by epithelial and/or endothelial cells may facilitate release of angiogenic growth factors locally from ECM.

In summary, we have established a novel, physiologically relevant *in vitro* model system that not only recapitulates several important aspects of estrogen-induced growth and preneoplastic progression of MCF10AT1 cells *in vivo*, but also demonstrates for the first time the integral role endothelial cells play in ductal-alveolar morphogenesis and proliferation of preneoplastic human breast epithelial cells. This assay system will provide a unique tool to explore systematically the expression of growth regulatory molecules that determine epithelial-specific and endothelial-specific requirements for angiogenesis and progression of preneoplastic breast disease. Defining the factors and cellular interactions that influence proliferation, invasion, cytological and functional differentiation of mammary epithelial and stromal components such as endothelial cells is key to development of effective intervention strategies for breast cancer.

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Figure 1. Phase contrast morphology of cells in 3-D Matrigel culture. (a and b) MCF10A cells at 24 h and 10 days, respectively; (c and d) EIII8 cells at 24 h and 10 days, respectively; (e and f) HUVEC cells at 24 h and 5 days, respectively. Note the difference in contrast on day 10 between MCF10A (b) and EIII8 (d) cultures that is produced by multilayering of epithelium. Bar 100 μm.

Figure 2. Phase contrast morphology of EIII8 cells in 3-D Matrigel culture following treatment with estrogen. (a) Cultures treated with 100 nM ICI 182,780; (b and d), cultures treated with 1 or 10 nM 17 β-estradiol (E_2), respectively; (c), cultures treated with a combination of 1 nM E_2 and a 100-fold molar excess of ICI 182,780. All cultures represent morphologies at 5 days of culture. Note the remarkable differences in contrast between cultures exposed to ICI 182,780 (a and c) and E_2 (b and d). Also, note that addition of ICI 182,780 significantly inhibits epithelial multilayering induced by E_2. Treatment with E_2 also induce formation of several new central spaces and connecting bridges (b and d). Bar 100 μm.

Figure 3. Phase contrast morphology of heterotypic EIII8-HUVEC or MCF10A-HUVEC co-cultures in Matrigel at day 5. MCF10A-HUVEC or EIII8-HUVEC cultures were treated with vehicle (0.01 % ethanol, v/v; panels a and d, respectively), 1 nM E_2, (b and e, respectively), or a combination of 1 nM E_2 and a 100-fold molar excess of ICI 182,780 (c and f, respectively). Note the difference in the sizes of "endothelial cell enriched spots" (⇦) formed on EIII8- and MCF10A-epithelia. Also, note the specific association of "ductal-alveolar outgrowths" (⬅) with "endothelial cell enriched spots" in EIII8-HUVEC cultures (d and e), and the specific inhibition by ICI 182, 780 of ductal-alveolar growth and endothelial cell enriched spots in EIII8-HUVEC

(panel f) and endothelial cell growth in MCF10A-HUVEC cultures (panel c). The inset in panel f demonstrates the results of exposure to ICI 182, 780 for 14 days. Bar 100 μm.

Figure 4. Phase contrast micrographs of 3-D EIII8-HUVEC co-cultures. (a and b), co-cultures established with DiI- and DiO-prelabeled EIII8 and HUVEC cells, respectively. Note that ductal-alveolar outgrowths are comprised of epithelial cells (a) whereas DiO-labeled endothelial cells are concentrated at this region as a spot (b). Note the presence of immature buds at day 5 of culture (c and d) that have developed into distinct and well formed buds by day 10 (e and f). The dark areas in close association with alveolar structures represent the endothelial enriched sites. Bar 100 μm (a,b,c,e) and 50 μm (d and f).

Figure 5. Regulation of 3-D growth of EIII8-HUVEC cultures by estrogen. Growth was quantitated by MTS assay on day 5 of culture. Control wells received additions of vehicle (0.01% ethanol (v/v). Treatments included E_2 at 0.1, 1 or 10 nM; ICI 182, 780 at 100 or 1000 nM; and combinations of 1 nM E_2 with 100-fold or 1000-fold excess of ICI 182,780. Results obtained from three independent experiments performed in triplicate are expressed as mean ± S.E. * Indicates doses of compounds that increased cell growth significantly over non-hormone-treated control ($P < 0.01$). **Indicates doses of ICI 182,780 that significantly decreased cell growth induced by E_2 ($P<0.001$).

Figure 6. Effect of conditioned medium (CM) from MCF10A-HUVEC, EIII8-HUVEC or EIII8 cultures on proliferation of HUVEC cells. HUVEC cells were treated with 50 μl or 1-20 μl of

unconcentrated CM from EIII8, MCF10A-HUVEC, or EIII8-HUVEC cultures, respectively. Effects of antibodies on CM-induced HUVEC cell proliferation were tested in cultures treated with combination of 20 μl of CM from EIII8-HUVEC cultures and 10 μg/ml of anti-VEGF, anti-Flk-1 or anti-IL-8 antibodies, or an equivalent amount of mouse or rabbit normal IgG. Since no differences in cell numbers were observed between cultures treated with mouse or rabbit normal IgG, the results are grouped together. Results obtained from three independent experiments performed in triplicate are expressed as mean ± S.E. * Indicates cell proliferation that is significantly increased by CM from EIII8-HUVEC co-cultures over untreated control ($P<0.001$). ** Indicates significant decrease in cell proliferation caused by addition of anti-VEGF or anti-Flk-1 antibodies relative to cultures treated with normal IgG ($P<0.001$). ***Indicates significant decrease in cell proliferation induced by anti-IL-8 antibody as compared to cultures treated with normal IgG ($P<0.02$).

Figure 7. Formalin-fixed paraffin-embedded sections of EIII8-HUVEC 3-D cultures were either stained with H&E (a) or with antibodies to PCNA (b), cytokeratins (c), muc-1 (d), cd31 (e), or Factor VIII (f). Although staining was done on serial sections, the sections look different. This inherent difficulty in obtaining identical serial sections is contributed by the small size and irregular growth of invading branching buds in Matrigel. Note the presence of numerous proliferating cells (b) in the finger-like projections or branching buds that are invading into the surrounding ECM with coincident ECM degradation (a and b). Also, note the widespread immunoreactivity to cytokeratins (c) as compared to the localized distribution of cd31-positive (e; ➤) and Factor VIII-expressing cells (f; ←). MUC-1 staining (d) is predominantly localized in

the lumen of epithelium either polarized to apical membrane of luminal epithelial cells or distributed in the cytoplasm (⇦). X10 (a,b,c,e and f) and X 4 (d).

Figure 8. 3-D EIII8-HUVEC co-cultures show upregulated expression of $VEGF_{165}$, Flk-1, ER, IL-8, and MMP-2. 25 μg of protein present in culture media and matrix fractions were analyzed by Western blotting.

(A) $VEGF_{165}$ levels were analyzed in culture media of 5 day-old homotypic (EIII8 or MCF10A), heterotypic (EIII8-HUVEC or MCF10A-HUVEC) cultures, or in HUVEC cells at 48 h of culture. (B) Levels of $VEGF_{165}$ secreted into culture media from EIII8-HUVEC co-cultures were analyzed on days 1, 5 and 10 of culture. Culture media were collected at indicated times from cultures treated with vehicle (0.01% ethanol, v/v), 1 nM E_2 or a combination of 1 nM E_2 and a 100-fold molar excess of ICI 182, 780. Position of $VEGF_{165}$ is indicated by an arrow in panels A and B.

(C) Steady-state levels of Flk-1 and ER, or IL-8 were analyzed in matrix and corresponding culture media, respectively, from HUVEC cultures on day 2 (lane 1), MCF10A-HUVEC (lanes 2 and 3), or EIII8-HUVEC (lanes 4-9) cultures on days 5 (lane 2, and lanes 4-6) and 10 (lane 3, and lanes 7-9). Cultures were treated with vehicle (lanes 1- 4 and 7), 1 nM E_2 (lanes 5 and 8), or with a combination of 1 nM E_2 and a 100-fold molar excess of ICI 182,780 (lanes 6 and 9). Positions of Flk-1, wild type ER, 42 kDa ER-reactive band and IL-8 are indicated.

(D) Zymographic analysis of gelatinases secreted from HUVEC (lane 2), EIII8 (lane 3), MCF10A (lane 4), EIII8-HUVEC (lane 5) and MCF10A-HUVEC (lane 6) cultures. Lane 1 represents activated MMP-2 used as positive control. Conditioned media prepared from 5 day-

old EIII8, MCF10A, EIII8-HUVEC or MCF10A-HUVEC cultures, or at 2 days of HUVEC cell cultures were analyzed on gelatin-embedded substrate gels. The gels are representative of three independent experiments.

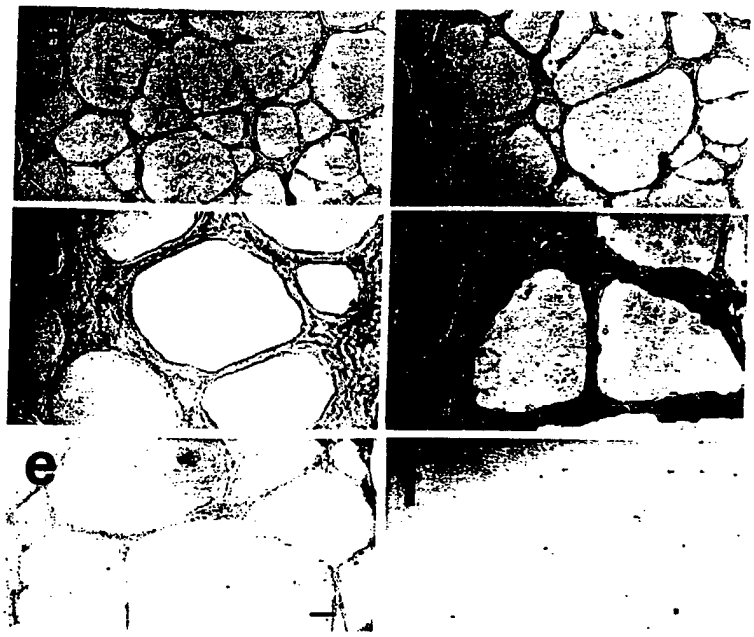


Figure 1

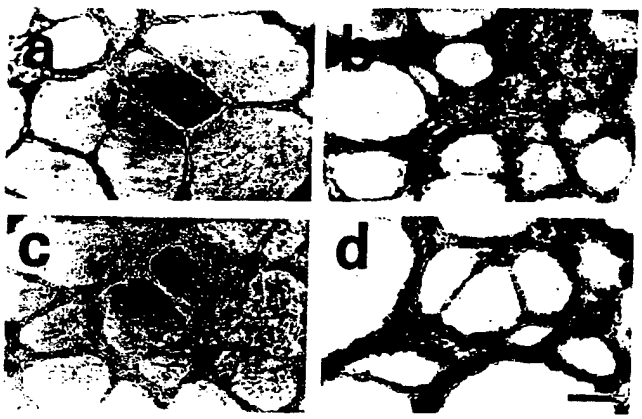


Figure 2

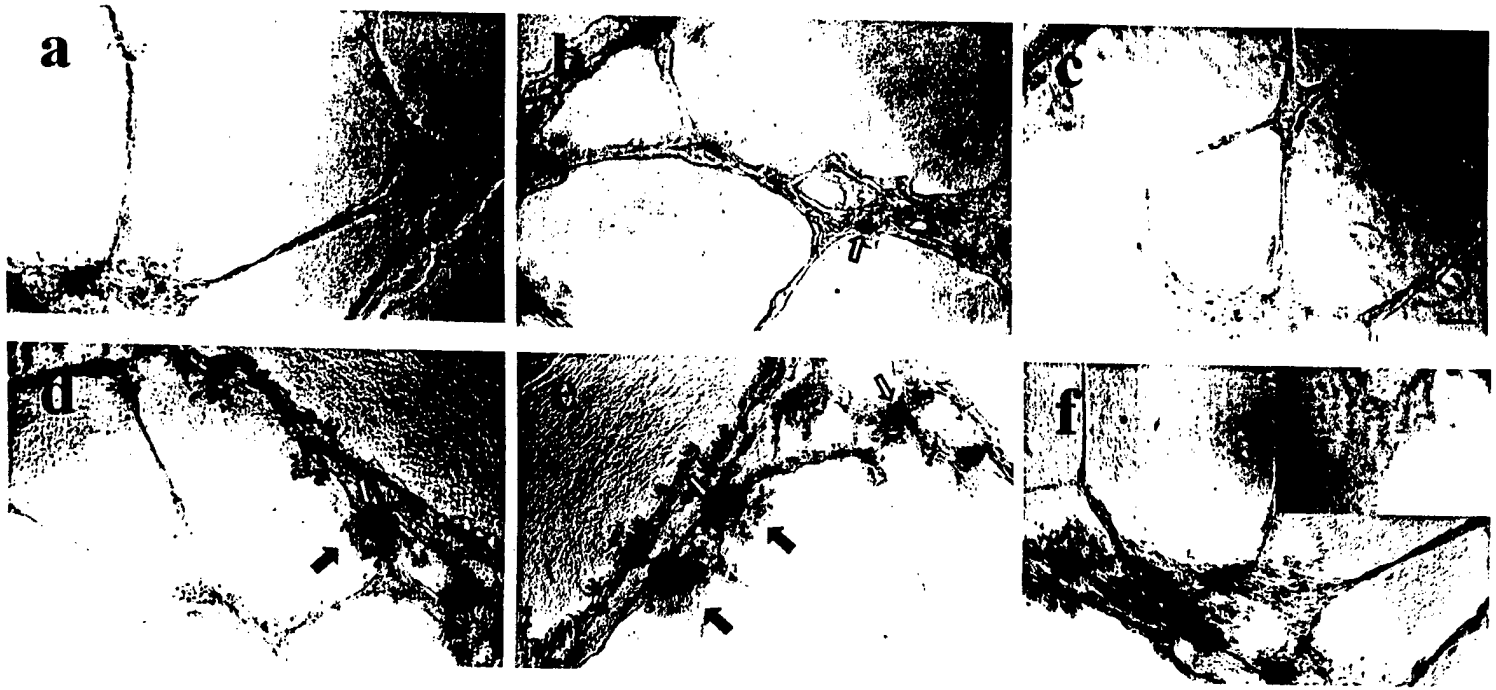


Figure 3

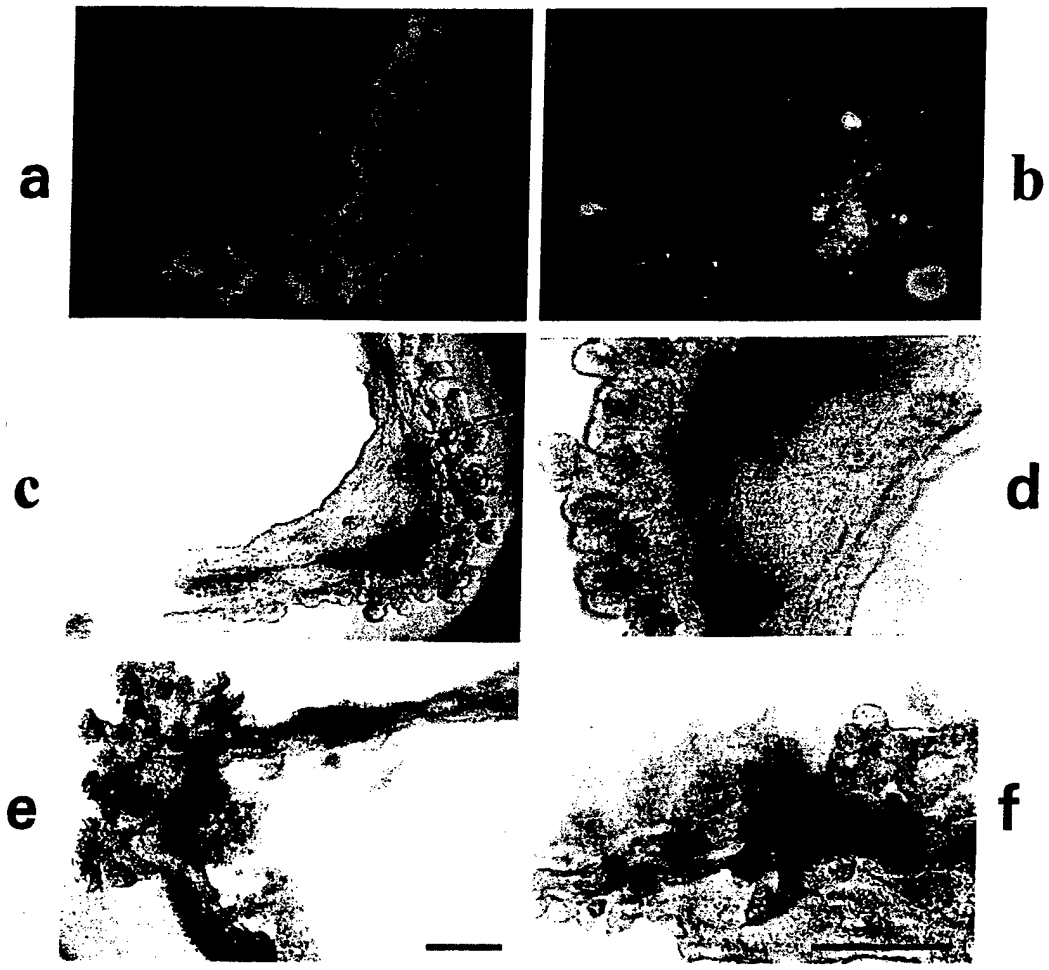
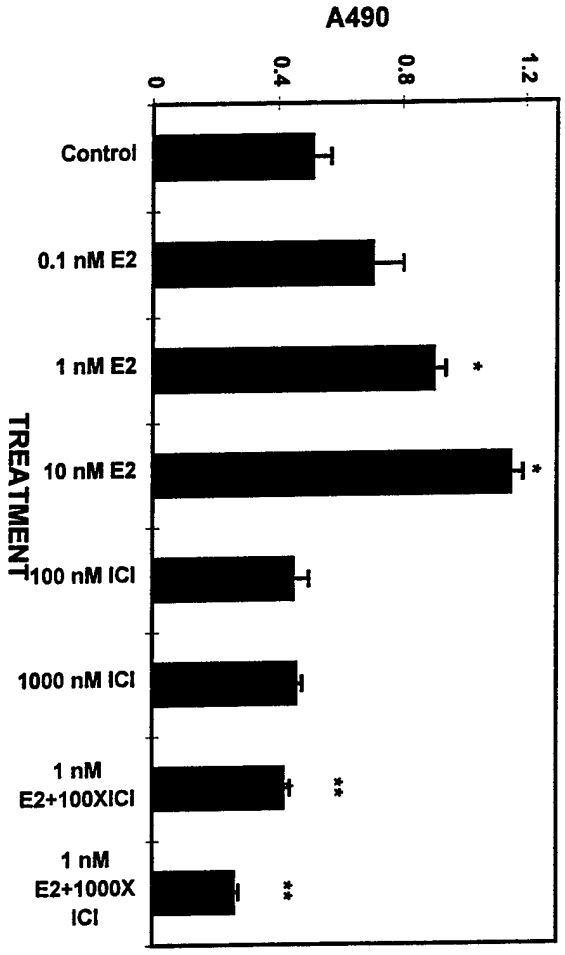


Figure 4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1 nM E2+1000X ICI
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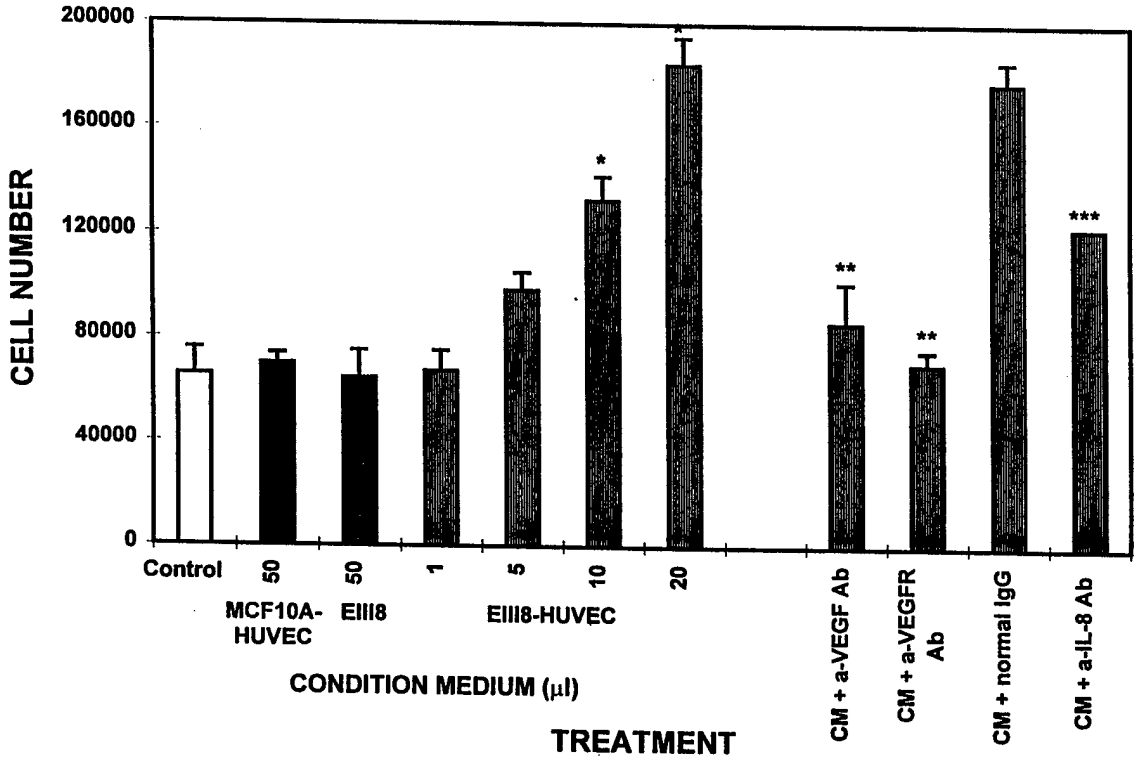
Figure 5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EIII8-HUVEC
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CM + a-VEGFR Ab
CM + normal IgG
CM + a-IL-8 Ab
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Figure 6

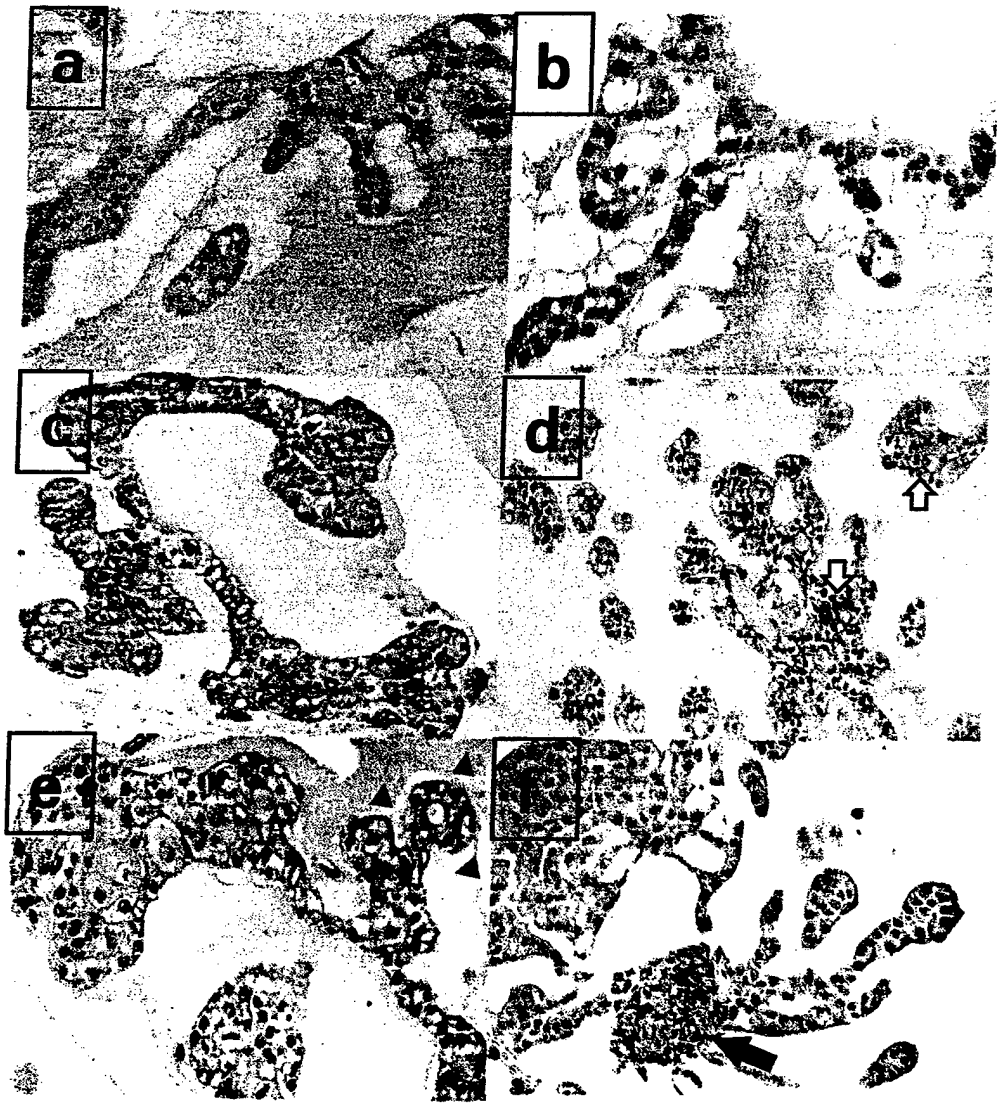

Figure 7

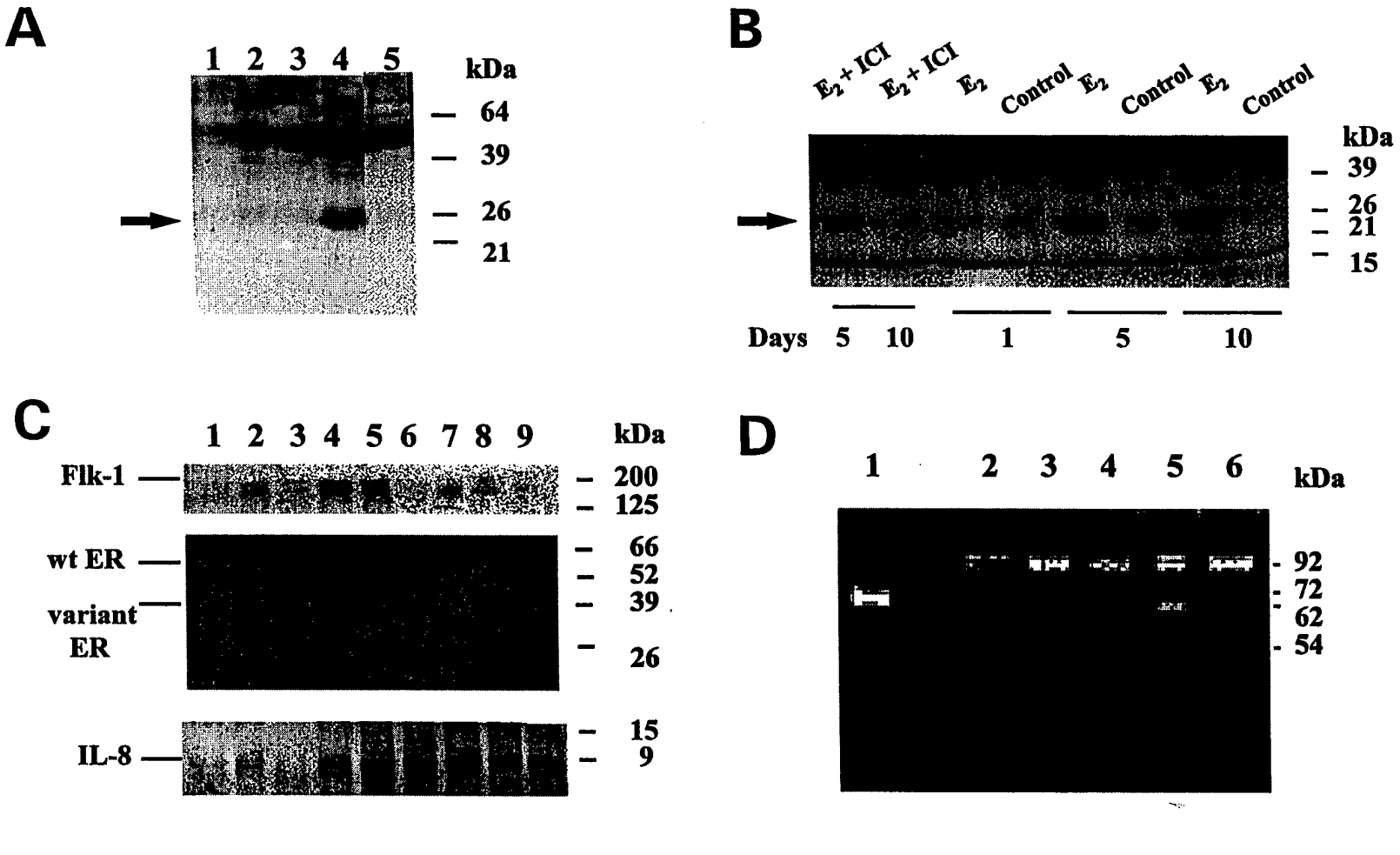
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Figure 8